PATENT
Attorney Docket No. A-57496/DJB

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:) Examiner: ZISKA, S.
)
WEISS, et al.) Group Art Unit: 1804
)
Serial No. 07/961,813)
)
Filed: 16 October 92)
)
For: REMYELINATION USING NEURAL STEM CELLS)

CERTIFICATE OF MAILING

I hereby certify that this correspondence, including listed enclosures, is being deposited with the United States Postal Service as First Class Mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, DC 20231 on ~~MARCH 29, 1994~~. 31 May 1994. vh 31 May 94

Signed: Vicki L. Henry 31 May 94
Vicki L. Henry

DECLARATION UNDER 37 C.F.R. §1.132

Commissioner of Patents
and Trademarks
Washington, DC 20231

Sir:

The undersigned, Joseph P. Hammang, hereby declares and states that:

1. I am currently working as a scientist at CytoTherapeutics Inc. in Providence, Rhode Island. My Curriculum Vitae is attached hereto.

2. I am a co-inventor of the captioned application and I have read the arguments in the outstanding Official Action, dated December 2nd 1993, wherein the Examiner rejected Claims 1-17 under 35 U.S.C. § 101 because evidence has not been presented to show that the invention works as claimed.

3. I have diligently performed or supervised experiments that show that neural stem cells isolated from donor tissue can be proliferated in a culture

medium containing a growth factor to produce precursor cells. The precursor cells can be harvested and transplanted into a myelin-deficient recipient wherein the precursor cells can differentiate into oligodendrocytes and remyelinate the axons of the recipient.

4. In these experiments, multi-potential EGF-responsive stem cells were isolated from the striata of E14-15 rats and mice and propagated in a defined, serum-free medium containing 20 ng/ml EGF. Nestin positive cells (i.e. no mature oligodendrocytes) were collected and triturated into a single cell suspension in the presence of 0.1% BSA. This suspension was concentrated to 50,000 cells/μl and one microliter of cells was injected into the dorsal columns of the T13/L1 region of the spinal cord of myelin deficient rats. Those animals transplanted with the mouse stem cells received Cyclosporin A at a dose of 10mg/Kg ip for the duration of the experiment.

5. Thirteen or fourteen days after injection, the animals were euthanized by perfusion fixation, the spinal cords removed and the tissue examined by light and electron microscopy. Patches of myelin were found in the dorsal columns of the recipients of both rat and mouse cells, indicating that neural stem cells isolated from rat and mouse neural tissue can differentiate into oligodendroglia and are capable of myelination *in vivo*.

6. The methods described in paragraphs 4 and 5 above are substantially the same as those disclosed in Example 2 (p. 17 to 18 of the specification) with only minor changes in the procedure (e.g. the rats were allowed to survive 2 weeks post-injection rather than 3 weeks; the rats were treated with antibiotics post-injection).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that willful, false statements may jeopardize the validity/enforceability of the application or any patent issued thereon.

Dated: 3/31/94

Signature: Joseph P. Hammang

Joseph P. Hammang, Ph.D.

Curriculum Vitae

JOSEPH P. HAMMANG
3 Prospect Street
Barrington, RI 02806
(401) 246-1893

Employment:

Principal Scientist /Assoc. Dir. Neuro & Molecular Biology)
May 1993 - Present
CytoTherapeutics, Inc., Providence, Rhode Island
Scientist - (June 1992 - May 1993)

Research Investigator I - CNS Cell and Molecular Neurobiology
Bristol-Myers Squibb Company
Pharmaceutical Research Institute,Wallingford, Connecticut
December 1991 - May 1992

Postdoctoral Fellow - CNS Cell and Molecular Neurobiology
Bristol-Myers Squibb Company
Pharmaceutical Research Institute, Wallingford, Connecticut
January 1991 - December 1991

Ph.D. Research Assistant - University of Wisconsin
School of Veterinary Medicine
Department of Pathobiological Sciences, Madison, Wisconsin
January 1988 to January 1991

Associate Researcher - University of Wisconsin
School of Veterinary Medicine
Department of Medical Sciences, Madison, Wisconsin
March 1987 - January 1988

Specialist - Life Sciences Research
University of Wisconsin - School of Veterinary Medicine
Department of Medical Sciences, Madison, Wisconsin
May 1983 - March 1987

Education:

Ph.D. - University of Wisconsin - Madison
Department of Veterinary Science (area of Neuroscience)

Master of Science - Zoology
June 1983, University of Wisconsin-Oshkosh

Bachelor of Science
May 1980, University of Wisconsin-Oshkosh
Major: Biology with emphasis in Zoology

Professional Honors:

Recipient of University of Wisconsin-Madison
Academic Staff Professional Development Grants
Years 1985/86 and 1986/87

Teaching Assistantships:

Taught lab and discussion of course entitled "Human Biology" - an introductory biology course with a human perspective for Nursing, Psychology, Social Work and Physical Education majors. Spring 1982

Taught lab and discussion of course entitled "Man in the Biosphere" - Fall 1981 - an introductory level biology course, ecologically oriented for non-majors.

Related Experiences:

Research Assistantship in Biology. Worked in electron microscopy lab. School year 1980-1981.

Biology internship with Wisconsin Department of Natural Resources, Oshkosh. Worked as assistant in Warm Water Fisheries Research Unit. Included radio telemetry, cartography and fish management techniques. Interned 12 weeks.
Summer of 1980.

University of Wisconsin-Oshkosh Department of Biology. Worked as lab assistant in setting up labs and reagent mixing for animal and human physiology courses. September 1979 to May 1980.

University of Wisconsin-Oshkosh Department of Chemistry. Worked as lab preparation assistant. September 1978 to May 1979.

University of Wisconsin-Fond du Lac. Worked as lab preparation assistant in Chemistry. September 1977 to August 1978.

Professional Memberships:

Society for Neuroscience
Sigma Xi
AAAS
American Society for Cell Biology

Activities:

College - Inducted as associate member of Sigma Xi
Scientific Research Society of America, Spring 1983
President of Iota Phi Chapter of Beta Beta Beta Biological Honor Society 1979-1980
Member of UW-Oshkosh Biology Club 1978-1982

Honors and Achievements:

Honor roll three semesters UW-Oshkosh

Community:

Volunteer as counselor for Badger Boys State Ripon, Wisconsin - June of 1975, 1976 and 1977.

Volunteer as co-advisor for environmental and ecology post of Explorer Scouts, Oshkosh, Wisconsin. Conducted tours and field trips - May 1980 to May 1982.

Workshop leader for "College for Kids" program (Neurobiology), University of Wisconsin-Madison, Summers of 1986, 1987, 1988, 1989, and 1990.

Research Skills:

Primary neuronal culture preparation; Mammalian cell culture; DNA transfection - primary cells and cell lines; Immuno-cytochemistry and immunohistochemistry; Electron microscopy and specimen preparation; Autoradiography; Histological preparations; CNS microinjection; Western blotting; Northern blotting; In situ hybridization; Production of rabbit polyclonal antisera; Photomicroscopy and micrograph preparation.

Research Symposium Presentations:

Presented preliminary research for M.S. thesis at the 18th annual Lake Superior Biological Conference, September 25-26, 1981 at Marquette, Michigan. Title: Ultrastructural anatomy of the urinary bladder of the freshwater teleost, Lota lota (Linnaeus).

Presented preliminary results of Masters research at the American Society of Zoologists annual meeting, December 27-30, 1982 at Louisville, Kentucky. Title: Morphology and ultrastructure of the urinary bladder of the burbot, Lota lota (Linnaeus).

Presented final results of the M.S. thesis research at the American Fisheries Society, Wisconsin Chapter meeting, March 11-12, 1983 at the Institute of Paper Chemistry in Appleton, Wisconsin. Title: Ultrastructural evidence for the physiological function of the urinary bladder of the burbot, Lota lota (Linnaeus).

The nervous system beyond paraffin. I.D. Duncan and J.P. Hammang. Co-presented to the National Histology Society, Region IV Meeting, Milwaukee, Wisconsin, June 1984.

The Shaking Pup: A canine central nervous system myelin mutant. I.D. Duncan, J. Hammang, K. Lord, and K. Dyer. Presented to the American College of Veterinary Pathologists and the Annual Meeting of the American Society of Veterinary Clinical Pathologists. Toronto, Ontario, Canada, November 1984.

The effect of tellurium on intraspinal axons myelinated by Schwann cells. J.P. Hammang, Presented at the Upper Midwest Peripheral Nerve Meeting, Mayo Clinic, Rochester, MN. November 1984.

Light microscopic and ultrastructural studies on the equine recurrent laryngeal nerve. I.D. Duncan and J.P. Hammang, University of Wisconsin-Madison. Presented to the Peripheral Nerve Study Group Meeting, September 9-12, 1985, Murren, Switzerland.

Identification of animal cells and cellular structures using specialized immunocytochemical techniques. J.P. Hammang. Presented to the Wisconsin Histology Society, Madison, Wisconsin, May 1986.

Presented preliminary Ph.D. research at the Mouse Molecular Genetics Conference, Cold Spring Harbor Laboratory, August 1988.

Invited Reviews:

E.E. Baetge, J.P. Hammang, V.K. Gribkoff and K.F.Meiri. The role of GAP-43 in the molecular regulation of axon outgrowth and electrophysiological properties. Perspectives on Developmental Neurobiology, 1992, 1(1), p.21-28.

Immortalized cell lines from targeted oncogene expression in transgenic mice. J.P. Hammang, E.E. Baetge, A. Messing. NeuroProtocols, 1993, 3 p. 176-183.

Referred Publications:

Peripheral Neuropathy in two dogs: Correlation between clinical, electrophysiological and pathological findings. K.R. Dyer, I.D. Duncan, J.P. Hammang and R.R. Dubielzig. J. of Small Animal Practice 1986, 27, 133-146.

Degenerative changes in rat intraspinal Schwann cells following tellurium intoxication. J.P. Hammang, I.D. Duncan and S.A. Gilmore. Neuropathol. and Appl. Neurobiol. 1986, 12, 359-370.

Myelin deficient rat: analysis of myelin proteins. K. Yanagisawa, I.D. Duncan, J.P. Hammang and R.H. Quarles. J. Neurochem., 1986, 47, No. 6, 1901-1907.

Myelin mosaicism in female heterozygotes of the canine shaking pup and myelin deficient rat mutants. I.D. Duncan, J.P. Hammang and K.F. Jackson. Brain Res., 1987, 402, 168-172.

Node-like axonal undercoating in the optic nerve of heterozygous myelin deficient rats. W.F. Blakemore, I.D. Duncan and J.P. Hammang. Brain Res., 1987, 403, 361-365.

Ultrastructural observations of organelle accumulations in the equine laryngeal nerve. I.D. Duncan and J.P. Hammang. J. Neurocytology, 1987, 16, No. 2, 269-280.

Subclinical entrapment neuropathy of the equine suprascapular nerve. I.D. Duncan, R.K. Schneider and J.P. Hammang. ACTA Neuropathol., 1987, 74, 53-61.

Abnormal compact myelin in the myelin deficient rat; absence of proteolipid protein corresponds with a defect in the intraperiod line. I.D. Duncan, J.P. Hammang and B.D. Trapp. Proc. Nat. Acad. Sci. (USA), 1987, 84, 6287-6291.

Transplantation of oligodendrocytes and Schwann cells into the spinal cord of the myelin deficient rat. I.D. Duncan, J.P. Hammang, K.F. Jackson, P.M. Wood, R.P. Bunge and L. Langford. J. Neurocytology, 1988, 17, 351-360.

Schwann cell myelination of the myelin deficient rat spinal cord following x-irradiation. I.D. Duncan, J.P. Hammang and S.A. Gilmore. Glia, 1988, 1, No. 3, 233-239.

Proliferation of rat intraspinal Schwann cells following tellurium intoxication. J.P. Hammang, S.F. Worth, I.D. Duncan and S.A. Gilmore. Acta Neuropathol., 1988, 76, 624-627.

Myelination in the jimpy mouse in the absence of proteolipid protein. I.D. Duncan, J.P. Hammang, S. Goda and R.H. Quarles. Glia, 1989, 2, 148-154.

Hypomyelination in the neonatal rat central and peripheral nervous systems following tellurium intoxication. K.F. Jackson, J.P. Hammang, S.F. Worth and I.D. Duncan. Acta Neuropathol., 1989, 78, 301-309.

Immortalized retinal neurons derived from SV40 T-antigen-induced tumors in transgenic mice. J.P. Hammang, E.E. Baetge, R.R. Behringer, R.L. Brinster, R.D. Palmiter and A. Messing. Neuron, 1990, 4 (5), 775-782.

Neurite outgrowth in PC12 cells deficient in GAP-43. E.E. Baetge and J.P. Hammang. Neuron, 1991, 6 (1), 21-30.

Immortalized retinal neurons used as immunogen for the generation of cell-specific antisera. J.P. Hammang and A. Messing. Brain Research, 1991 556, 85-94.

Phenylethanolamine N-methyltransferase (PNMT)-expressing horizontal cells in the rat retina: a study employing double-label immunohistochemistry. J.P. Hammang, M.C. Bohn and A. Messing. J. Comp. Neurol., 1992, 316, 383-389.

Po promoter directs expression of reporter and toxin genes to Schwann cells of transgenic mice. A. Messing, R.R. Behringer, J.P. Hammang, R.D. Palmiter, R.L. Brinster, and G. Lemke. Neuron, 1992, 8, 507-520.

Oncogene expression in retinal horizontal cells of transgenic mice results in a cascade of neurodegeneration. J.P. Hammang, R.R. Behringer, E.E. Baetge, R.L. Brinster, R.D. Palmiter and A.Messing. Neuron, 1993, 10, p. 1197-1209.

Development of myelin mosaicism in the optic nerve of heterozygotes of the X-linked myelin-deficient rat mutant (*md*). I.D. Duncan, K.F. Jackson, J.P. Hammang, D. Marren, R. Hoffman. Developmental Biology, 1993, 157, p. 334-347.

Immortalization of embryonic rat hippocampal cells following infection by an amphotropic retrovirus containing the adenoviral E1A gene. R.J. Robbins, E.E. Baetge, D.L. Needels, G. P. Dotto and J.P. Hammang, (submitted).

The mouse neurofilament-L promoter confers neuron-specific expression of a ß-galactosidase reporter gene in primary neuronal and glial cell cultures. J.P. Hammang, C.M. Sampson, D.L. Needels and E.E. Baetge, (submitted).

Derivation of adrenergic adrenal chromaffin cell lines from the targeted expression of a PNMT-SV40 fusion gene. J.P. Hammang et al., (in preparation).

Hypomyelinating peripheral neuropthies and Schwannomas in transgenic mice expressing SV40 T-antigen. A. Messing, R. R. Behringer, L. Wrabetz, J. P. Hammnag, G. Lemke, R. D. Palmiter and R. L. Brinster. J. Neuroscience (1994) (in press).

ECF-Responsive neural stem cells isolated from rat and mouse brain can form myelin following transplantation into the myelin-deficient rat CNS. J. P. Hammang, D. R. Archer, and I. D. Duncan (1994) (In preparation).

Polymer encapsulated cells genetically modified to secrete human nerve growth factor promote the survival of oxotomized septal cholinergic neurons. S. R. Winn, J. P. Hammang, D. F. Emerich, A. Lee, R. D. Palmiter and E. E. Baetge. 1994, PNAS March.

Delivery of a putative Parkinson's factor (GDNF) into the rat CNS using apolymer-encapsulated cell line. J. P. Hammang, D. F. Emerich, S. R. Winn, A. Lee, M. D. Lindner, M. Schinstine adn E. E. Baetge. (In preparation).

Reduced electrical excitability of PC 12 cells deficient in GAP-43: comparison with GAP-43-positive cells. V. K. Gribkoff, J. P. Hammang, and E. E. Baetge, 1994 (submitted).

Mutagensis of serine[41] to alnine inhibits the association of GAP-43 with the membrane skeleton of GAP-43- deficient PC12 β cells: Effects on cell adhesion, and the composition of neurite cytoskeleton and membrane. K. F. Meiri, J. P. Hammang, E. W. Dent, and E. E. Baetge. J. Cell Biol. 1994 (submitted).

Published
Abstracts:

1. An immunocytochemical investigation of myelin proteins in the Shaking Pup I.D. Duncan, J.P Hammang and B. Trapp. J. Neuropathol. and Exp. Neurol., 1984, 43, 328.

2. The effect of elemental tellurium (Te) on rat intraspinal Schwann cells. J.P. Hammang, I.D. Duncan and S.A. Gilmore. J. Neuropathol. and Exp. Neurol., 1985, 44, 332.

3. Subclinical entrapment neuropathy of the equine suprascapular nerve. J.P. Hammang and I.D. Duncan. J. Neuropathol. and Exp. Neurol., 1986, 65, 370.

4. Mosaicism in the CNS of the myelin mutants, the shaking pup and the myelin deficient (md) rat. I.D. Duncan, J.P. Hammang and K.F. Jackson. J. Neuropathol. and Exp. Neurol., 1986, 65, 383.

5. Heterozygotes of the shaking pup and myelin deficient (md) rat mutants show mosaicism in the CNS. I.D. Duncan, J.P. Hammang and K.F. Jackson. Abstracts of X International Congress on Neuropathology, Stockholm, Sweden, September 1986.

6. Peripheral neuropathy in SV40 transgenic mice: Immunohisto-chemical studies of myelin proteins. A Messing, J.P. Hammang, I.D. Duncan, C.A. Pinkert, R.D. Palmiter and R.L. Brinster. Society for Neuroscience, 1986 Abstracts, 12, p. 264.

7. Glial cell division in the optic nerve of the myelin deficient rat. K.F. Jackson, J.P. Hammang and I.D. Duncan. Society for Neuroscience, 1986 Abstracts, 12, p. 1584.

8. Schwann cell myelination in the myelin-deficient rat spinal cord. I.D. Duncan, J.P. Hammang and S.A. Gilmore. Society for Neuroscience, 1986 Abstracts, 12, p. 160.

9. Ultrastructural changes in granulosa cells of Chrysemys picta after a single injection of LH-RH. I.Y. Mahmoud, R.V. Cyrus, M.J. Woller and J.P. Hammang. American Zoologist, 1986, 26, No. 4, 3A.

10. Transplantation of Schwann cells and oligodendrocytes into the spinal cord of the myelin deficient rat. I.D. Duncan, J.P. Hammang, K.F. Jackson, L. Langford, P.M. Wood and R.P. Bunge. J. Neuropathol. and Exp. Neurol., 1987, 46, 351. (Honorable mention, Weil Award for best research paper.)

11. Proliferation of intraspinal Schwann cells in tellurium intoxicated rats. J.P. Hammang, I.D. Duncan, S.F. Worth and S.A. Gilmore. J. Neuropathol. and Exp. Neurol., 1987, 46, 357.

12. Jimpy myelin lacks PLP and has a defect in the intraperiod line. I.D. Duncan, J.P. Hammang and K.F. Jackson. Society for Neuroscience, 1987 Abstracts, 13, p. 118.

13. Tellurium-induced hypomyelination in the neonatal rat central and peripheral nervous systems. J.P. Hammang, S.F. Worth and I.D. Duncan. Society for Neuroscience, 1987 Abstracts, 13, p. 698.

14. Further observations on the glial cell population of the optic nerve of the myelin deficient rat. K.F. Jackson, J.P. Hammang and I.D. Duncan. Society for Neuroscience, 1987 Abstracts, 13, p. 885.

15. Myelin mosaicism in the optic nerve of the myelin deficient rat heterozygote. I.D. Duncan, J.P.Hammang, K.F. Jackson and S.F. Worth. J. Neuropathol. and Exp. Neurol., 1988, 47, p. 384.

16. Derivation of neuronal cell lines from retinal and adrenal tumors in PNMT-SV40 transgenic mice. J.P. Hammang, R.R. Behringer, E.E. Baetge, R.D. Palmiter, R.L. Brinster and A. Messing. Society for Neuroscience, 1988 Abstracts, 14, p. 473.

17. Schwann cell-specific gene expression in transgenic mice. A. Messing, R.R. Behringer, J.P. Hammang, G. Lemke, R.L. Brinster and R.D. Palmiter. J. Neuropathol. and Exp. Neurol., 1989. (Weil Award for best research paper.)

18. An immortalized line of retinal neurons derived from PNMT-SV40 transgenic mice. J.P. Hammang, E.E. Baetge, R.R. Behringer, E.P. Sandgren, R.D. Palmiter, R.L. Brinster and A. Messing. Society for Neuroscience, 1989 Abstracts, 15, p. 1396.

19. PC12 cells devoid of GAP-43 protein synthesize a truncated form of GAP-43 mRNA. E.E. Baetge, C.M. Sampson and J.P. Hammang. Society for Neuroscience, 1990 Abstracts, 16, p. 812.

20. Electrophysiological properties of GAP-43-containing and GAP-43-negative PC12 cells exposed to NGF. V.K. Gribkoff, E.E. Baetge and J.P. Hammang. Society for Neuroscience, 1990 Abstracts, 16, p. 813.

21. Distribution of myelin patches in the mosaic optic nerves of the myelin deficient rat heterozygote. I.D. Duncan, J.P. Hammang, K.F. Jackson, D. Marren, and C. Iida. Society for Neuroscience, 1990 Abstracts, 16, p. 665.

22. The C6 glioma cell line expresses the growth-associated protein GAP-43 in a developmentally regulated fashion. J.P. Hammang, A. Messing and E.E. Baetge. Society for Neuroscience, 1990 Abstracts, 16, p. 812.

23. Transgenic ablation of Schwann cells during development. A. Messing, G. Lemke, R.R. Behringer, J.P. Hammang, R.D. Palmiter and R.L. Brinster. Society for Neuroscience, 1990 Abstracts, 16, p. 1167.

24. Altered organization of intermediate filament proteins in GAP-43 deficient PC12(B) cells. J.P. Hammang, K.F. Meiri and E.E. Baetge. Society for Neuroscience, 1991 Abstracts, 17, p. 1310.

25. Abnormal neurite extension in GAP-43 deficient PC12 cells stably transfected with mutated (SER^{41}-ALA^{41})GAP-43. E.E. Baetge, L.E. Bickerstaff, K.M. Felsenstein, J.P. Hammang and K.F. Meiri. Society for Neuroscience, 1991 Abstracts, 17, p. 1309.

26. Phenylethanolamine N-methyltransferase-expressing adrenal chromaffin cell lines derived from PNMT-SV40 transgenic mice. A. Messing, E.E. Baetge and J.P. Hammang. Society for Neuroscience, 1991 Abstracts, 17, p. 38.

27. Characterization of the mouse neurofilament L (NF-L) promoter in primary neuronal cell cultures. J.P. Hammang, C.M. Sampson, D.L. Needels and E.E. Baetge. J. Cell Biol. 1991 115(3), Part 2, p. 396a.

28. Trophic effects of neurotrophin-3 on hippocampal neurons. D.L. Needels, M.E. McGuire, S.B. Roberts, K.M. Ingalls and J.P. Hammang. Society for Neuroscience, 1992 Abstracts, 18, p. 44.

29. Trophic effects of neurotrophin-3 on astrocytes in mixed cell cultures from fetal rat hippocampus. M.E. McGuire, D.L. Needels, S.B. Roberts, K.M. Ingalls and J.P. Hammang. Society for Neuroscience, 1992 Abstracts, 18, p. 44.

30. EGF-generated mouse striatal neurospheres express the trk neurotrophin receptor. J.S. Williams, A. Vescovi, B.A. Reynolds, J.P. Hammang, E.E. Baetge, and S. Weiss. Society for Neuroscience, 1992 Abstracts, 18, p. 217.

31. GAP-43 is developmentally regulated in glial cells derived from EGF responsive CNS stem cells. J.P. Hammang, B.A. Reynolds, E.E. Baetge and S. Weiss. Society for Neuroscience, 1992 Abstracts, 18, p. 217.

32. Targeted oncogenes in neuronal and neuroendocrine cells using the PNMT promoter in transgenic mice. J.P. Hammang, E.E. Baetge and A. Messing. International Society for Developmental Neuroscience, June 1992.

33. Oncogene expression in retinal horizontal cells of transgenic mice results in a cascade of neurodegeneration. J.P. Hammang, R.R. Behringer, E.E. Baetge, R.D. Palmiter, R.L. Brinster and A. Messing. Cold Spring Harbor Laboratory, Mouse Molecular Genetics, August 1992.

34. Transplantation of encapsulated PC12 cells into non-human primate brain. B. R. Frydel, M. J. Banks, V. Hovanesian, S. R. Winn, S. D. Sherman, D. F. Emerich, P. E. McDermott, B. J. Dean, J. P. Hammang, F. Gentile, E. J. Doherty and E. E. Baetge. Soceity for Neuroscience, 1993 Abstracts, 19, p. 56.

35. Development of a polymer-encapsulated cellular implant for pain modulation. S. R. Winn, S. D. Sherman, S. A. Morrison, J. Harvey, A. Lee, J. Sagen, J. P. Hammang, and E. E. Baetge. Soceity for Neuroscience, 1993 Abstracts, 19, p. 1410.

36. Release of CNTF from encapsulated transfected BHK cells and from polymer rods. S. A. Tan, V. Padurn, A. C. Kato, A. D. Zurn, R. Palmiter, B. Bamber, J. P. Hammang, E. E. Baetge, P. Aebischer. Society for Neuroscience, 1993 Abstracts, 19, p. 197.

37. Long-term expression of nerve growth factor from polymer encapsulated cells in the rat CNS. J. P. Hammang, B. J. Dean, A. Lee, D. F. Emerich, S. R. Winn, B. Bamber, R. D. Palmiter, and E. E. Beatge. Society for Neuroscience, 1993 Abstracts, 19, p. 657.

38. CNTF, LIF and oncostatin M increase the number of oligodendrocytes in cultures of EGF-generated CNS progenitor cells. B. A. Reynolds, J. P. Hammang, E. E. Baetge and S. Weiss. Society for Neuroscience, 1993 Abstracts, 19, p. 870.

39. The delivery of neurotrophic factors to the nervous system using polymer encapsulated cells. E. E. Baetge, S. R. Winn, A. Lee, B. J. Dean, B. Bamber, R. D. Palmiter and J. P. Hammang. Soceity for Neuroscience, 1993 Abstracts, 19, p. 657

40. ECF- responsive neural stem cells isolated from rat and mouse brain are capable of differentiating into oligodendrocytes and of forming myelin following transplantation into the myelin deficient rat. I. D. Duncan, D. R. Archer and J. P. Hammang. Society for Neuroscience, 1993 Abstracts, 19, p. 689.

41. Generation of a transformed cell-line with a neuronal phenotype from RIPTag transgenic mouse insulinoma. D. Fiore, D. Knaack, C. R. Greco, O. D. Hegre, and J. P. Hammang. American Society for Cell Biology, 1993 Abstracts, 4, p. 375a.

42. Delivery of putative Parkinson's factor (GDNF) into the rat CNS using a polymer-encapsulated cell line. E. E. Baetge, D. F. Emerich, S. R. Winn, A. Lee, M. D. Lindner and J. P. Hammang. American Society for Cell Biology, 1993 Abstracts, 4, 442a.

43. EGF-responsive neural stem cells derived from MBP-*lac* Z transgenic mice can express the transgene in differentiating oligodendrocytes. J. P. Hammang, L. G. Wrabetz, J. Kamholz and A. Messing. American Society for Cell Biology, 1993 Abstracts, 4, p. 374a.

NHL-009/US-8

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

PATENTS

Examiner	:	Suzanne Ziska
Group Art Unit	:	1804
Applicants	:	Weiss et al.
Serial No.	:	08/479,796
Filed	:	June 7, 1995
For	:	REMYELINATION OF NEURONS USING MULTIPOTENT NEURAL STEM CELL PROGENY

Hon. Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION

I, JOSEPH P. HAMMANG, hereby declare and state as follows:

1. I am one of the named co-inventors of the above-identified application.

2. I received my B.S. and M.S. (Zoology) degrees from the University of Wisconsin, Oshkosh, Wisconsin in 1980 and 1982, respectively. I received my Ph.D. (Neuroscience) from the University of Wisconsin, Madison, Wisconsin in 1990. I am currently employed at CytoTherapeutics, Inc., a licensee of certain rights in this invention. I have been working in the field of cell and molecular neurobiology and with myelin mutant animals since 1982, and working with neural stem cells since 1991.

3. I have read the above-identified patent application, and am aware of the Examiner's August 19, 1997 Office Action. I understand that the pending claims are 16-18, 32-34, 36-46, 48-56 and 58-63.

4. I understand that the Examiner has rejected claims 16-18, 32-34, 36-46, 48-56, and 58-63 under 35 U.S.C. § 112, first paragraph, contending that the specification is not enabling for remyelination. In particular, I understand that the Examiner contends that "[f]orming patches of myelin are different than forming myelin around the axon ... which implies that the myelin sheath has been reformed." I also understand that the Examiner contends that "[a]pplicants have failed to disclose evidence that the injection of neural cells into any and all demyelinated axons would comprise any type of treatment for any and all types of demyelination." I further understand that the Examiner further contends that the specification "fails to disclose guidance relating to the amount of cells to inject, the site of injection, longevity of cellular existence, and longevity of myelin expression."

5. I also understand that the Examiner has rejected claim 16 under 35 U.S.C. § 112, first paragraph, contending that the recitation that the cells have not been treated with serum *in vivo* is not enabled since serum is a natural constituent of blood. Applicants' attorneys have informed me that the *in vivo* recitation was a typographical error, and was intended to read "*in vitro*" – accordingly, I have not discussed this further, as I understand it has been amended in the accompanying response.

6. I understand that the Examiner has rejected claims 40 and 41 under 35 U.S.C. § 112, first paragraph, contending that "the specification is not enabling for remyelination of neurons in primates and/or humans."

7. I also understand that the Examiner has maintained her rejection of claims 16 and 18, 34, 36-43, 45, 46, 48-53, 55, 56 and 58-63 under 35 U.S.C. § 103, contending that those claims are obvious over Boyles taken with Hunter, Gage, and Masters.

8. I further understand that the Examiner has maintained the rejection of claims 17, 32, 33, 44 and 54 under 35 U.S.C. § 103, contending that the claims are unpatentable over Boyles taken with Hunter, Gage, and Masters, in further view of Morrison.

9. I make this declaration to rebut the Examiner's rejections, and to report results obtained by workers under my supervision or by collaborators at my request.

The 35 U.S.C. § 112, first paragraph rejections

10. The Examiner has rejected claims 16-18, 32-34, 36-46, 48-56, and 58-63 under 35 U.S.C. § 112, first paragraph, contending that the specification is not enabling for remyelination. In particular, I understand that the Examiner contends that "[f]orming patches of myelin are different than forming myelin around the axon ... which implies that the myelin sheath has been reformed." (Office Action, p. 2, emphasis added). I also understand that the Examiner contends that "[a]pplicants have failed to disclose evidence that the injection of neural cells into any and all demyelinated axons would comprise any type of treatment for any and all types of demyelination." I further understand that the Examiner further contends that the specification "fails to disclose guidance relating to the amount of cells to inject, the site of injection, longevity of cellular existence, and longevity of myelin expression."

11. As I understand the Examiner's comments regarding "patches of myelin", I believe that the Examiner is referring to the data presented in Example 15, which states that "[p]atches of myelin were found in the dorsal columns of the recipients of both rat and mouse cells, indicating that neural stem cells isolated from rat and mouse neural tissue can differentiate into oligodendroglia and are capable of myelination *in vivo*." Spec., p. 69, lines 22-25.

12. I would like to clarify the data reported in Example 15. That example demonstrated that transplanted proliferating neurospheres (clusters of cells containing neural stem cells) appropriately differentiate into oligodendrocytes when implanted into a myelin deficient environment. That experiment also demonstrated that upon differentiation, those neural stem cell-derived oligodendrocytes actually produced myelin *in vivo*. Most importantly, the patches of myelin that we observed were in fact patches of appropriately myelinated axons with myelin sheaths wrapped around them – as called for in the pending claims.

13. We have performed additional experiments in both small and large mammal models of myelin deficiency. Our data further demonstrate that neural stem cell derived-oligodendrocytes do not just form aberrant myelin-like structures, but, in fact, form myelin around the axon, definitively shown by electron microscopy. This data directly addresses the Examiner's rejection.

Rodent Stem Cell Transplantation

14. In one series of experiments, we transplanted undifferentiated rat or mouse neural stem cell progeny into the spinal cord of a central nervous system ("CNS") myelin mutant, the myelin-

deficient ("*md*") rat. The *md* rat is characterized by a failure of development and early death of oligodendrocytes. The *md* rat CNS is virtually devoid of myelin. We sought to determine whether these neural stem cell progeny were capable of differentiating *in vivo* into oligodendrocytes and myelinating axons. This work is published in Hammang et al., "Myelination Following Transplantation of EGF-Responsive Neural Stem Cells into a Myelin-Deficient Environment", Exp. Neurol., 147, pp. 84-95 (1997) (copy attached as Ex. 1 hereto).

15. Briefly, we first prepared neural stem cell cultures from wild type E14-15 Sprague-Dawley rats or BalbC mice, as described in the specification. See, e.g., Example 1 and Example 4, p. 55, lines 11-18; p. 56, lines 19-30. These cultures were passaged once per week for up to 25 passages, as described in the specification. See, e.g., Example 6, p. 57, line 19 - p. 58, line 3.

16. For implantation, nestin-positive neurosphere clusters (with no detectable oligodendrocytes) were harvested, triturated into a single cell suspension and concentrated to about 50,000 cells/μl. Transplantation of the neural stem cell suspension was substantially as outlined in Example 15 of the specification. Recipient *md* rats were anaesthetized, and a laminectomy was performed to expose the T13-L1 level of the spinal cord. About 1 μl (i.e., about 50,000 cells) of the neural stem cell suspension was injected into the dorsal columns lateral to the midline. Animals were sacrificed about 12-14 days after the transplants. Ex. 1, p. 86, left column.

17. The transplanted stem cell progeny formed myelin *in vivo*. Visualization of intact spinal cords revealed that myelination was evident over 3 mm rostral to caudal in some cases. Toluidine blue-stained transverse sections of spinal cords two weeks after transplantation

revealed extensive areas of myelin within the dorsal columns. See, Ex. 1, p. 89 left column, Figs. 2A-D. In some of the implanted animals, myelinated fibers were seen throughout the dorsal columns extending as deeply as the margin of the corticospinal tracts (Ex. 1; p. 89 left column, Fig. 2E).

18. We also examined the ultrastructural characteristics of the *md* rat spinal cords that had received stem cell progeny implants. Numerous mature oligodendrocytes were seen elaborating myelin sheaths around the *md* axons. It is important to note that such oligodendrocytes and myelin sheaths are not seen in the uninjected *md* rat or away from the transplant site. Ex. 1, p. 90, left column. Formation of myelin sheaths around axons in these animals was only seen after transplantation of neural stem cell cultures according to the claimed invention.

19. Importantly, Panel A and Panel B of Ex. 1, Fig. 5 shows electron micrographs of nonmyelinated axons away from the transplant site in the *md* rat (Panel A) compared to a transplanted area in which the majority of axons are myelinated, with myelin formed around the axon. This myelination was of normal compaction, possessing both major and minor dense lines. Ex. 1, p. 90. No ectopic neurons or evidence of gliosis was observed.

20. We concluded that undifferentiated, nestin-positive neurosphere cultures which lacked expression of glial markers prior to transplantation, upon transplantation into a myelin-deficient environment, gave rise to mature myelin-producing oligodendrocytes capable of ensheathing multiple axons with apparently ultrastructurally normal myelin. Both rat and mouse stem cell cultures reproducibly produced this result.

21. These *in vivo* results were unexpected in view of the *in vitro* results obtained with the rat and mouse stem cell cultures. When induced to proliferate *in vitro*, the majority of the cells in the neurosphere cultures form astrocytes. Ex. 1, p. 89, right column. Because of this finding, it was possible that astrogliosis would occur *in vivo*. As noted above, we did not see any such gliosis – to the contrary, our data suggest that in this myelin-deficient environment, there was appropriate oligodendrocyte differentiation, as well as formation of ultrastructurally normal myelin, of normal compaction, possessing both major and minor dense lines. Ex. 1, p. 90, right column.

Canine Stem Cell Transplantation

22. In a second series of experiments, my collaborators isolated and characterized canine neurospheres (cultures containing neural stem cell progeny). Undifferentiated canine neural stem cell progeny were then transplanted into the spinal cord of another myelin mutant, the shaking ("*sh*") pup, as well as the ("*md*") rat. The *sh* pup model, like the *md* rat model, arises from an exonic point mutation in the proteolipid protein gene. The mutation arose spontaneously in a colony of Welsh springer spaniels and causes severe tremor from about 12 days after birth. The central nervous system ("CNS") is severely hypomyelinated, with reduced numbers of normal, mature oligodendrocytes. These experiments sought to determine whether canine neural stem cell progeny were capable of differentiating *in vivo* into oligodendrocytes and forming myelin around axons after transplantation. This work is published in Milward et al., "Isolation and Transplantation of Multipotential Populations of Epidermal Growth Factor-Responsive, Neural

Progenitor Cells from the Canine Brain", J. Neurosci. Res., 50, pp. 862-71 (1997) (copy attached as Ex. 2 hereto).

23. Canine neural stem cell cultures were prepared from wild type embryonic day 40 to post-natal day 8 donor pups, substantially according to the methodology described in the specification for murine stem cell cultures. See, e.g., spec. p. 55, lines 11-18; p. 56, lines 19-30, and Ex. 2, p. 863. These cultures were expanded *in vitro* for at least 6 months, according to the methodology described in the specification. See, e.g., spec., p. 57, line 19 - p. 58, line 3, and Ex. 2, p. 865.

24. Populations of proliferative canine neurospheres were transduced to express lac Z (as a marker). Ex. 2, p. 863, right column.

25. The transduced canine neurospheres were transplanted into the spinal cords of a postnatal 14 day and an adult (7 months) *sh* pup. Transduced donor cells had undergone 3 and 9 passages, respectively, prior to transplant. Recipient pups were anaesthetized, and a laminectomy was performed at thoracic and lumbar sites T13-L1, L1-2, and L2-3. The canine neural stem cell suspension (at a concentration substantially as outlined in the specification, Example 15) was slowly injected into the spinal cords using a micromanipulator and Hamilton syringe. Ex. 2, p. 864, right column. The postnatal pup was sacrificed at 6 weeks and the adult pup at 2 weeks after transplant.

26. After X-Gal staining, transverse sections of the *sh* spinal cord in the regions of the injection sites revealed distinct blue clusters (indicating remyelination) at the site of implantation. Blue staining was also observed in the lateral and ventral columns and gray matter, suggesting migration of implanted cells. Light microscopy of the tissue sections clearly

demonstrated *lacZ*-expressing cells in association with those areas containing myelin. See, Ex. 2, p. 868, right column. Microscopy also confirmed that the grafted cells had integrated normally into the adult *sh* pup cytoarchitecture. See, Ex. 2, p. 867, right column.

27. Canine neurospheres (transduced to express lac Z) were also transplanted into the dorsal columns of spinal cords of 7 day postnatal *md* rats at the thoracic-lumbar (T13-L1) junction. At 11 days posttransplant, blue X-gal reaction product was observed spread up to 6 mm along the dorsal midline of at least three transplant recipients in each of two separate experiments. Transverse sections of recipient spinal cords revealed grafted cells in the dorsal columns in areas containing myelinated axons. Ex. 2, p. 868. As I noted above, the *md* rat CNS is normally virtually devoid of myelin. Strikingly, a grafted progeny cell with a long cytoplasmic process in contact with a myelinated axon was observed. Ex. 2, p. 868 and Fig. 1M.

28. This data from the canine neurosphere transplants shows that neural stem cell progeny grafts can survive at least 6 weeks in postnatal and at least 2 weeks in adult *sh* pup recipients (allogeneic). The data also demonstrate that the neural stem cell progeny grafts can myelinate axons under xenograft conditions, as seen in the *md* rat transplants.

29. The foregoing data, in my opinion, demonstrate that mammalian neural stem cell cultures are capable of remyelination of axons in both small mammal and large mammal myelin-deficiency models.

30. I note that the transplantation protocol followed for these experiments was substantially identical to that detailed in the specification (p. 68, line 16 - p. 70, line 7), which provides specific guidance as to the cell suspension concentration, the cell numbers used for these

transplants, and the site of injection. Further, in my opinion, given the comprehensive specification, and its specific guidance, the ordinarily skilled artisan with specification in hand, has more than sufficient teaching to transplant neural stem cell cultures into a variety of mammalian species, including humans, for remyelination without undue experimentation.

31. The Examiner has questioned whether injection of neural cells into certain non-myelinated axons is useful as a treatment for "any and all" types of myelin-deficiency disorders. I do not think that this concern addresses the claimed invention. Rather, it appears that the Examiner's concern as to the type of myelin-deficiency disorder focuses on the cause for the myelin-deficiency. The cause of the myelin-deficiency, while interesting for developing prophylactic therapies, is not relevant in this invention. This invention seeks to remyelinate non-myelinated axons regardless of the cause of their myelin-deficiency. I note that the claims recite a method of providing neural stem cell progeny to form myelin around the axon of a neuron. We (or our collaborators at our direction) have unequivocally demonstrated that upon transplantation of neural stem cell progeny, myelin does in fact form around the axon of neurons – exactly as recited in the pending claims.

32. I should further note that the remyelination we have observed according to the claimed invention was not abnormal – to the contrary, we observed ultrastructurally normal myelin, of normal compaction, possessing both major and minor dense lines, formed fully around multiple axons. This is an important result, since formation of an appropriate myelin sheath is believed necessary for achieving the nerve conduction velocities found in wild type animals. I have attached as Ex. 3 a copy of a paper entitled "Transplantation of Glial Cells Enhances Action

Potential Conduction of Amyelinated Spinal Cord Axons in the Myelin-deficient Rat", Proc. Natl Acad. Sci USA, 91, pp. 53-57 (1994) published by Dr. Ian Duncan's group (our collaborator in the *md* rat and *sh* pup studies described above). Dr. Duncan's paper demonstrates that axons remyelinated by transplanted cells do not have impaired frequency-response properties, and that cell-transplant-remyelinated axons exhibit conduction velocities approaching normal values.

Primate/Human Neurosphere Cultures

33. I understand that the Examiner has rejected claims 40 and 41 under 35 U.S.C. § 112, first paragraph, contending that "the specification is not enabling for remyelination of neurons in primates and/or humans." As I understand the Examiner's rejection, it focuses on remyelination of neurons in recipient primates and/or humans. I believe that the Examiner's rejection is improper, as it appears that the Examiner has misunderstood the subject matter recited in claims 40 and 41. Those claims recite that the source of the mammalian neural tissue (containing neural stem cells) be derived from primates and/or humans – not that the recipient be a primate or human. There is no question that the specification provides more than adequate guidance to isolate and culture primate and human neural stem cell cultures from primate or human neural tissue. Example 10 (p. 64, line 14 - p. 65, line 7) refers specifically to isolation of primate neurosphere cultures from adult Rhesus monkey neural tissue, and Example 9 (p. 62, line 11 - p. 64, line 12), Example 11 (p. 62, line 11 - p. 64, line 12) and Example 14 (p. 67, line 27 - p. 68, line 15) refer specifically to isolation of human neurosphere cultures from human neural tissue. This is precisely what is recited in claims 40 and 41.

34. Additional work using human cell counterparts to the foregoing rodent and canine neurosphere cell cultures has been published in Cattaneo et al, "Non-Virally Mediated Gene Transfer Into Human Central Nervous System Precursor Cells", Mol. Brain Res., 42, pp. 161-66 (1996) (copy attached as Ex. 4 hereto). These multipotent human cell cultures have been serially subcultured and expanded for several years in serum-free growth medium containing epidermal growth factor (EGF), and, like their rodent and canine homologues, grow in suspension forming "neurosphere" aggregates of variable sizes. Also like their rodent and canine homologues, the human neurosphere cultures are capable of differentiating into neurons and glia (including oligodendrocytes) upon removal of the growth factor mitogen from the culture medium. It is also my opinion that, since the mechanism of myelination in mammals is thought to be substantially the same from species to species, demonstration of remyelination in the rodent and canine models is reasonably predictive for other mammalian species, including primates and humans.

35. Accordingly for the reasons I have discussed above, I believe that the ordinarily skilled artisan with the specification in hand could practice the claimed invention to form myelin around an axon using mammalian neural stem cell progeny, without undue experimentation.

The § 103 rejections

36. I understand that the Examiner has rejected claims 16 and 18, 34, 36-43, 45, 46, 48-53, 55, 56 and 58-63 under 35 U.S.C. § 103(a) over Boyles taken with Hunter, Gage, and Masters. I understand that the Examiner has maintained the rejection of claims 17, 32, 33, 44 and 54 under

35 U.S.C. § 103, contending that the claims are unpatentable over Boyles taken with Hunter, Gage, and Masters, in further view of Morrison.

37. Boyles is not relevant here – it does not refer to any neural stem cell cultures, or their use for remyelination. Boyles refers to accumulation of certain apolipoproteins in the regenerating and remyelinating mammalian peripheral nerve. The Examiner is correct in stating that it was known in the prior art that "oligodendrocytes play a pivotal role in the remyelination process". I, however, disagree with the Examiner's statement that "one of skill in the art would have been motivated to transplant cells capable of differentiating into oligodendrocytes". I disagree for several reasons. First, Boyles does not say one word about transplanting any cells, never mind neural stem cell progeny. Second, Boyles certainly does not teach or suggest the surprising results obtained according to this invention – namely that transplanting neural stem cell progeny into a myelin-deficient environment resulted in a preferential, and appropriately compensatory, differentiation along the oligodendrocyte lineage and production by those oligodendrocytes of ultrastructurally normal myelin. In fact, the myelin was of normal compaction, possessing both major and minor dense lines, formed fully around multiple axons, with no observable ectopic neuron production or evidence of gliosis (abundance of astrocytes or hypertrophied astrocyte processes).

38. Hunter does not remedy the deficiencies of Boyles or the other cited art. Hunter's cells are not multipotent as required by the pending claims; rather they are bipotent glial precursors committed to becoming astrocytes or oligodendrocytes. Hunter's cells lack one of the main advantages of the claimed invention – they cannot be cultured and expanded in serum free

medium in vitro to provide an adequate tissue source suitable for transplantation. As the Examiner notes, Hunter makes clear that the bipotent glial cells require live conditioning cells for survival. This raises tremendous regulatory concerns for transplants into humans because of the uncharacterized nature of conditioning medium (produced by the live conditioning cells). It also raises serious cell culture development concerns. Those concerns are avoided using the present invention.

39. And Hunter, alone or in combination with the other cited art, does not teach or suggest the surprising results obtained here; that the claimed invention results in appropriate oligodendrocyte formation, and production of ultrastructurally normal myelin, of normal compaction, formed fully around multiple axons, with no observable gliosis.

40. Gage is not relevant, does not teach or suggest the claimed invention, and does not cure the deficiencies of Boyles and Hunter or the other cited art. Gage refers to transplantation of genetically-modified cells. There is no requirement in the pending claims that the cells be genetically-modified in any way. All the experiments reported here relating to remyelination were performed with genetically unmodified cells – something not possible according to Gage. Nor does Gage refer to any multipotent neural stem cells or their progeny, as required by the instant claims. Finally, Gage, like the other cited art, simply does not teach or suggest the surprising results obtained here; appropriate oligodendrocyte formation and remyelination with ultrastructurally normal myelin, of normal compaction, formed fully around multiple axons, with no observable gliosis.

41. Similarly, Masters does not teach or suggest the claimed invention, alone or in combination with the other cited art. Masters refers to an *in vitro* culture system to induce bipotent glial progenitor cells to differentiate into oligodendrocytes. Masters does not say one single word about multipotent neural stem cell cultures and their progeny. Masters does not say one word about use of such cultures to remyelinate axons. And Masters, alone or in combination with the other cited art, does not teach or suggest the unexpected results obtained here – that transplanting neural stem cell progeny into a myelin-deficient environment resulted in appropriate oligodendrocyte differentiation, with production of ultrastructurally normal myelin, of normal compaction, formed fully around multiple axons, with no observable gliosis. In fact, in my opinion, Masters directs the ordinarily skilled artisan away from this invention and the unexpected results here, since Masters teaches a culture system for differentiating bipotent cells in vitro.

42. Finally, Morrison too is irrelevant. Morrison refers to use of epidermal growth factor ("EGF") as an elongation factor and maintenance factor for primary telencephalic neuron cultures derived from neonatal rat brain. Morrison does not say one word about neural stem cell cultures, and certainly not their use for remyelination, as recited in the pending claims. Nor does Morrison say one word about the surprising results obtained here; i.e., appropriate oligodendrocyte formation and remyelination with ultrastructurally normal myelin, of normal compaction, formed fully around multiple axons, with no observable gliosis. Accordingly, Morrison, taken alone or in combination with the other cited art, does not teach or suggest the claimed methods of remyelination.

43. The Examiner's comments relating to "a single definitive role for EGF" are not germane to the patentability of the pending method claims. Morrison's discussion of certain trophic effects of EGF on primary cultures of differentiated neurons has no bearing on its use as a mitogen for multipotent neural stem cell cultures, as described throughout the specification, and in the experiments described above. Importantly, Morrison says nothing about the claimed method of remyelination using neural stem cell cultures and the unique finding that remyelination according to the claimed method is ultrastructurally normal. Morrison, taken alone or in combination with the other cited art, simply does not lead the ordinarily skilled artisan to the claimed remyelination method.

44. For the foregoing reasons, I believe that the claims are patentable over the art that the Examiner has cited.

45. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001, Title 18, United States Code, and that willful false statements may jeopardize the validity of this application and any patent issuing therefrom.

Joseph P. Hammang

Signed at Lincoln, Rhode Island
this 11 day of February, 1998

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Stem Cells in the Central Nervous System

Ronald McKay

In the vertebrate central nervous system, multipotential cells have been identified in vitro and in vivo. Defined mitogens cause the proliferation of multipotential cells in vitro, the magnitude of which is sufficient to account for the number of cells in the brain. Factors that control the differentiation of fetal stem cells to neurons and glia have been defined in vitro, and multipotential cells with similar signaling logic can be cultured from the adult central nervous system. Transplanting cells to new sites emphasizes that neuroepithelial cells have the potential to integrate into many brain regions. These results focus attention on how information in external stimuli is translated into the number and types of differentiated cells in the brain. The development of therapies for the reconstruction of the diseased or injured brain will be guided by our understanding of the origin and stability of cell type in the central nervous system.

Definition of the processes that shape the cellular makeup of the central nervous system (CNS) has relied heavily on three distinct procedures: fate mapping, tissue culture, and transplantation. These traditional tools of embryologists have been significantly improved by the recent incorporation of advanced molecular methods. Fate mapping of neuronal precursors in vertebrates points to the existence of multipotential cells that are precursors to both neu-

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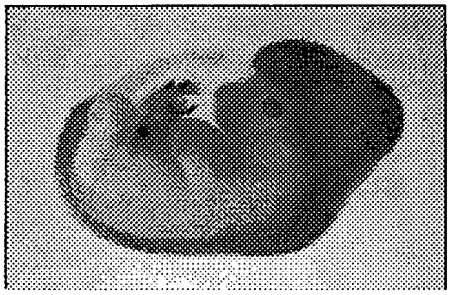

Fig. 1. A transgenic mid-gestation mouse fetus showing the expression (blue) in CNS stem cells of a reporter gene under control of 750 base pairs of the second intron of the nestin gene. The approach is described in detail in (*9*).

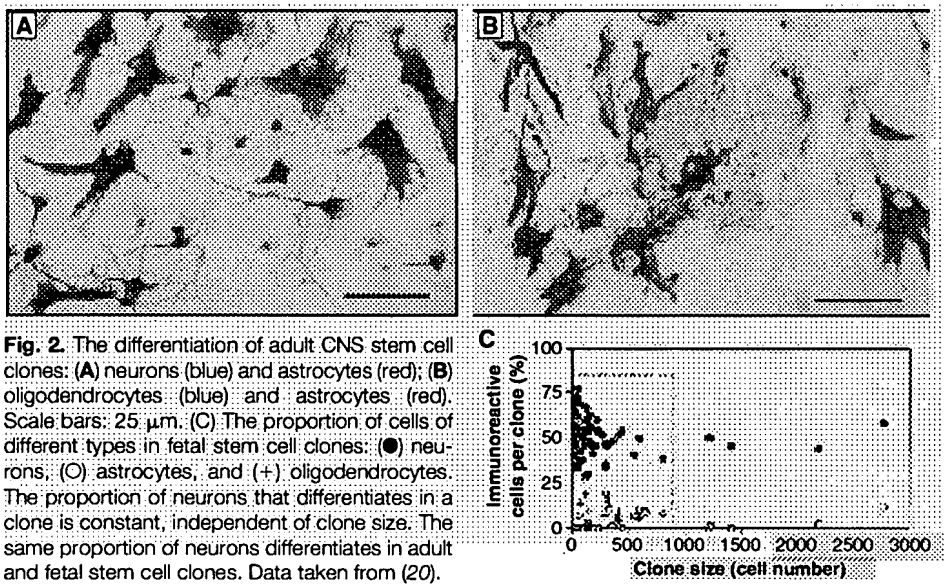

Fig. 2. The differentiation of adult CNS stem cell clones: (**A**) neurons (blue) and astrocytes (red); (**B**) oligodendrocytes (blue) and astrocytes (red). Scale bars: 25 μm. (C) The proportion of cells of different types in fetal stem cell clones: (●) neurons, (○) astrocytes, and (+) oligodendrocytes. The proportion of neurons that differentiates in a clone is constant, independent of clone size. The same proportion of neurons differentiates in adult and fetal stem cell clones. Data taken from (*20*).

rons and glia (*1*). However, this approach does not necessarily reveal the full proliferation and differentiation capability of the cells. In vitro and in vivo manipulations must be used to test the developmental potential of a cell. Tissue culture and transplant techniques, developed in vertebrate systems (*2*), have generated important data on the potential of neural cells (*3*).

Defining a Stem Cell

To be considered a stem cell in the CNS, a cell must have the potential to differentiate into neurons, astrocytes, and oligodendrocytes and to self-renew sufficiently to provide the numbers of cells in the brain. The term "progenitor" refers to a cell with a more restricted potential than a stem cell. "Precursor" is a less stringent term that refers to any cell that is earlier in a developmental pathway than another.

The complete cellular lineage of the nematode *Caenorhabditis elegans* has been described (*4*) and is an influential instance of the power of morphological analysis to define precursor-product relations in vivo. However, in the CNS of mammals, there are too many cells for each to be followed individually. The problem is similar to the technical difficulties biochemists faced in defining metabolic pathways. Without access to pure precursor, it was difficult to establish the catalytic step actually performed by a given enzyme. When this hurdle was overcome, it was recognized that enzymes perform discrete chemical steps, ultimately giving rise to the important concept of one gene–one enzyme (*5*). Similarly, to understand the developing brain, we need to purify the precursor cell types and define their transitions into differentiated progeny. Early work revealed that fetal cells removed from the developing brain and placed in vitro could give rise to differentiated neurons (*6*). For the most part, these neurons were derived from cells that did not divide in tissue culture, although cells that did divide could acquire some features of immature neurons (*7*).

The intermediate filament nestin is a major cytoskeletal protein in neuronal precursors in the mammalian CNS (*8*). Nestin is first detected at the earliest steps in neural plate induction (*9*), and most cells in the neuroepithelium are nestin-positive before neurogenesis (*10*) (Fig. 1). Coincident with their exit from the cell cycle, neurons down-regulate nestin and express distinct intermediate filaments. This transition has also been observed in vitro where precursor cells proliferate and differentiate into neurons (*11–16*) and glia (*17*).

Confirming the results of in vivo fate mapping, lineage experiments in vitro show that neurons and glia can be derived from a common fetal precursor cell (*12, 13, 15, 16, 18–20*) (Fig. 2). The adult nervous system also contains multipotential precursors for neurons, astrocytes, and oligodendrocytes (*13, 16, 18–20*). Cultured cells from both the fetal and adult CNS that have proliferated in vitro can differentiate to show morphological and electrophysiological features characteristic of neurons: regenerative action potentials and synaptic structures (*16, 21*) (Fig. 3). These data show the multipotential nature of cells derived from the CNS.

Quantitative studies have established the homogeneity and stability of multipotent cells derived from the fetal brain (*20*). In vitro these cells divide daily and efficiently generate neurons and glia for at least the first month of culture. These multipotent cells proliferate sufficiently in vitro to

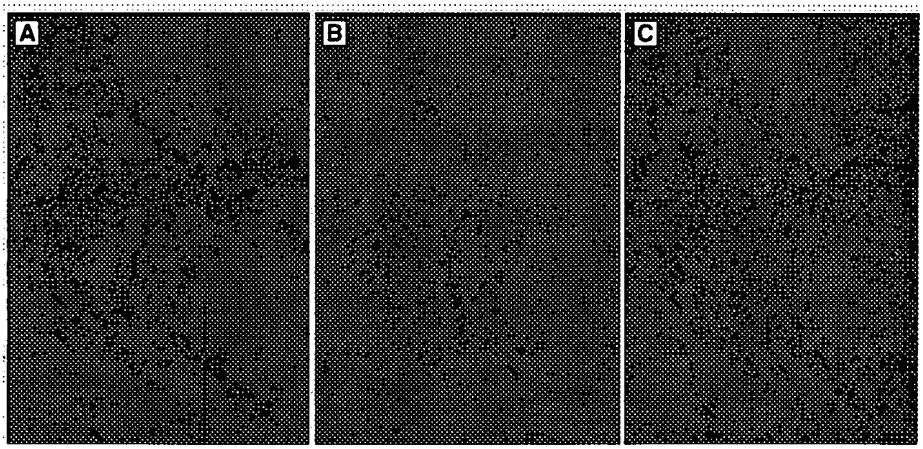

Fig. 3. Neuronal differentiation of CNS stem cells derived from the embryonic day-16 hippocampus. Cells were expanded for 16 days in the presence of bFGF followed by 21 days of differentiation in the presence of BDNF (20 ng/ml). (**A**) Staining with antibody to synapsin (green), (**B**) staining with antibody to MAP2, and (**C**) the two images superimposed. Synapsin is concentrated in presynaptic terminals, and MAP2, in dendrites. The culture and staining conditions are similar to those reported in (*21*).

account for the large numbers of cells present in the mammalian brain at birth (*10*). These cells can be considered to be stem cells because they fulfill the criteria of multipotency and self-renewal. Asymmetric division, which is sometimes considered to be a property of stem cells (*2*) and may actually occur in the neuroepithelium (*22*), does not appear to be necessary in cultured CNS stem cells (*20*).

Response Mechanisms and Transitions in Vitro

The extraordinary diversity of the adult vertebrate nervous system is generated from a sheet of epithelial cells over a period of several days. Precise numbers of neurons, astrocytes, and oligodendrocytes differentiate in successive waves. The spinal cord, formed from the caudal region of the neural tube, is one of the first sites of neuronal differentiation. Basic fibroblast growth factor (bFGF) is one mechanism that defines rostro-caudal identity in the neural tube (*23*). Neuronal differentiation in the dorsoventral axis is a response of uncommitted cells to successive extracellular signals (*24*). Sonic hedgehog and members of the transforming growth factor–β (TGF-β) family influence ventral and dorsal features of development in the caudal neural tube. These signals are used in several cellular contexts. For example, members of the TGF-β family influence segment-specific apoptosis in the neuroepithelium (*25*), astrocyte maturation (*26*), the differentiation of peripheral nervous system stem cells (*27*), and dorsoventral differentiation in the CNS (*24*). These diverse effects emphasize that the action of extracellular ligands depends on the integration of multiple signals by a specific responding cell.

Cell-autonomous mechanisms may also contribute to the generation of cell types in the nervous system. In the hematopoietic system, cell-autonomous stochastic processes are thought to generate all of the mature cell types, and the specificity of differentiation is a consequence of selective mechanisms (*28*). In such a system, specificity is obtained as a consequence of signals acting selectively only after the events that generate the different cell types. There is clear evidence for cell death in the neural tube (*29*) and growing knowledge of extra- and intracellular signals that mediate cell death (*30*). The high rates of apoptosis during neural development are consistent with an important role for selective mechanisms in the CNS.

Instructive mechanisms also occur in both the peripheral (PNS) and central nervous systems. Glial growth factor, a member of the heregulin-neuregulin class of factors, acts instructively on PNS stem cells to direct them to a Schwann cell fate (*31*). Bone morphogenetic proteins (BMP) 2 and 4 stimulate neurogenesis, and TGF-β1 generates smooth-muscle cells from the PNS stem cell (*27*). In the CNS, ciliary neurotrophic factor (CNTF) acts instructively on the multipotential stem cell, directing it to a committed astrocytic fate (*20*).

It has previously been shown that CNTF induces astrocytic differentiation in O2-A cells (*32*). In vitro, CNS stem cells rapidly and efficiently differentiate into astrocytes in the presence of CNTF (*20*). CNTF is not a mitogen for these cells, and a transient exposure (48 hours) to CNTF, even in the presence of mitogen, switches the differentiated state of more than 98% of the uncommitted stem cells. These data suggest that, in the absence of significant cell death, stimulating the Jak-stat system (the effector of CNTF) instructs the stem cell to become an astrocyte. In a recent study, BMPs promoted astrocytic differentiation from cells that had been expanded in vitro in the presence of epidermal growth factor (*26*). It will be interesting to establish whether BMPs and CNTF act through a common pathway at the same stage of astrocyte differentiation.

Thyroid hormone (T3) is also an instructive factor causing stem cells to become lineage-restricted progenitors for oligodendrocytes (*20*). Interestingly, CNTF and T3 are both differentiation and lineage-restriction factors. The differentiation of peripheral and central stem cells can be achieved without selection by the instructive action of extracellular signals. However, it seems likely that a combination of instruction and selection is used in vivo to precisely regulate precursor-product transitions at the cellular level.

The importance of selective mechanisms acting on a defined precursor cell type in brain development is best illustrated by studies on the differentiation of oligodendrocytes. There is evidence from the optic nerve for the existence of a bipotential progenitor in vitro for oligodendrocytes and type-2 astrocytes, the O-2A cell (*33*). In addition, the differentiation of this precursor cell could be controlled by manipulation of extracellular signals. Once the properties of this cell had been established, it became clear that a similar cell existed in the adult optic nerve (*34*). In tissue culture, O-2A cells respond to several factors [bFGF, platelet-derived growth factor, CNTF, neurotrophins, and T3], and many of these factors also act in vivo to increase oligodendrocyte number in the optic nerve (*35*). These in vivo data show that the availability of growth factors is limiting and that cell death is important in regulating oligodendrocyte numbers.

This summary indicates that simple ligands can regulate in vitro the transitions between stem cells and the three major cell types of the adult brain. However, it is not clear how many cell states exist in addition to stem cells and committed progenitors for astrocytes and oligodendrocytes. In some cases precursor-product transitions have been defined, but there are still many aspects of cell-type origins that are unclear and may be advanced by further work in vitro. Epidermal growth factor (EGF) and bFGF have both been used as mitogens to expand CNS stem cells, but EGF may not be the optimal choice for a stem cell mitogen, as there is evidence that EGF favors glial differentiation. In vitro EGF is a stem cell mitogen and a differentiation factor for astrocytes but not a lineage restriction factor, suggesting that the commitment event is distinct from the differentiation mechanism (*20*). However, the in vivo overexpression of EGF receptor may induce a fate shift from neurons to glia rather than simply promote astrocytic differentiation (*36*). It is clearly necessary to define the fundamental biochemical differences between lineage restriction in stem cells and differentiation of progenitor cells.

Another important unresolved question is whether there are proliferating cells capable of giving rise to specific kinds of neuron. There is evidence for a cell of this type in the postnatal cerebellum, but it is not clear whether a committed neuronal progenitor occurs in other brain regions (*37*). The events that generate the pluripotent CNS stem cell from an earlier totipotent embryonic stem cell can also be analyzed in vitro, because embryonic stem cells differentiate through a nestin-positive state to form synaptically active networks of central neurons (*38*). The routine differentiation of functional neurons from propagated stem cells would permit detailed analysis of how early steps in neurogenesis influence later stages of neuronal differentiation. The challenge is to set up experimental systems where the differentiation events of interest can be measured efficiently.

Space and Time

The cortical neuroepithelium is a highly polarized structure. Precursor cells divide at the inner (ventricular) surface of the neural tube, and immature neurons migrate away from the ventricle to specific layers. As different neurons become postmitotic in sequence, their laminar location is a function of the time when the neuron differentiated. Transplants in ferret cortex show that appropriate, layer-specific neuronal differentiation occurs when cells derived from an early time are moved to a later stage (*39*).

Conversely, late neuronal precursors transplanted to an earlier stage host do not contribute efficiently to early neuronal fates but rather exhibit laminar positions appropriate for late-generated neurons (*40*). This evidence supports a model where a neuron becomes committed to a particular laminar fate in the ventricular zone at the time of withdrawal from the cell cycle. Thus, it is the timing of the exit from the cycle that is thought to implement two distinct commitment events. In this scenario, the postmitotic neuron is locked into a specific fate, and the remaining precursors are also irrevocably changed.

It is not known what specifies the regional identity of the different areas of the CNS. There are complex patterns of expression of both cell surface signals and transcriptional regulators in the developing neuroepithelium long before neurons themselves differentiate (*41*). But there could be different stem cells for different brain regions. Gene deletion experiments in mice illustrate that whole sections of the brain can be eliminated with relatively little perturbation of the development of adjacent brain regions (*42*). Although these results are startling, they do not establish whether neuronal precursor cells are irreversibly committed to distinct regional fates. To establish commitment, we must give cells an opportunity to choose another regional fate. In the developing chick, a duplication of a brain region can be obtained by the local application of FGF8 (*43*). This result suggests that single factors are sufficient to bias the differentiation cascade and establish major regional features of the CNS.

The rhombomeres of the hindbrain are a good example of the compartmental arrangement of the neuroepithelium (*44*). Although it was first thought that cells were prohibited from crossing the boundaries between rhombomeric compartments, fate mapping in vivo now suggests that cells do move from one compartment to another at a low frequency (*45*). In other brain regions, neuronal precursors also migrate over great distances (*46*). When the location of rhombomeres was altered by tissue grafts, rhombomere-specific *Hox* gene expression was respecified by as yet undefined anterior-posterior control systems (*47*). In these transplant experiments, pieces of tissue were rearranged, making it hard to interpret the responses of single cells. It will be interesting to directly test the plasticity of isolated rhombomeric cells by transplanting dissociated cells from one rhombomere to another.

Grafting experiments with cell lines from the hippocampus support a model in which local signals in the neuroepithelium at the time of neurogenesis give rise to region-specific neuronal subtypes. Immortalized nestin-positive hippocampal cells transplanted to the developing cerebellum differentiated into typical cerebellar neurons (*48*). Transplants of primary striatal cells into the developing cerebral cortex also showed a switch to the locally appropriate fate (*49*), suggesting that the plasticity in cell fate shown with immortal cells was not an artifact of immortalization. In conceptually similar experiments, primary cerebellar cells derived from mice expressing the *lacZ* reporter gene under a neuron-specific promoter were grafted into the hippocampus of neonatal rats or wild-type mice. The grafted cells acquired morphological and immunohistochemical features of hippocampal granule neurons (*50*). The grafted and host neurons also showed kinetics of induction identical to those of the immediate early gene *c-fos* after intraperitoneal injection of neurotransmitter agonists and antagonists (*50*). These data suggest that immortal and primary neuroepithelial precursor cells grafted to new sites generate region-specific neurons in response to local cues.

A major limitation of postnatal transplantation studies was that heterotopic neuronal integration occurred efficiently only when donor cells were introduced into the few sites that continued to generate neurons in the newborn animal. This limited spectrum of accessible regions was dramatically increased by transplanting neural cells across the uterine wall into the embryonic mammalian brain (*49*, *51*, *52*). When genetically labeled mouse telencephalic neuroepithelial cells were simply deposited in the ventricles, large numbers of grafted cells were subsequently found incorporated into many sites in the host brain. The transplanted cells migrated in accordance with known pathways and incorporated into telencephalic, diencephalic, and mesencephalic regions (*52*). Surprisingly, cells derived from the dorsal and ventral forebrain incorporated into homotopic and many heterotopic brain regions in a similar fashion. After migration, the cells acquired morphological and antigenic features appropriate for neurons in their new environment (Fig. 4). The fact that striatal precursors can give rise to cortical, thalamic, and even tectal neurons illustrates that the regional heterogeneity of the brain results primarily from extracellular signals acting on precursors during neuronal migration and differentiation. These results indicate that the activation of different signaling pathways in uncommitted stem cells generates the spatial heterogeneity of neurons seen in the CNS.

Stem Cells and Disease in the Adult Nervous System

It is important to define the types of precursor cells that give rise to the neurons generated in the adult CNS (*53*, *54*). Cells from the adult brain proliferate and differentiate into neurons and glia in tissue culture (*13*, *14*, *16*, *55*) with the same efficiency for neuronal differentiation as found in fetal stem cells and the same responses to extracellular ligands (*20*). For example, 50% of the cells differentiate into neurons, and glial differentiation is strongly enhanced in response to CNTF and T3 in fetal and adult stem cells. Thus, similar general mechanisms control the differentiation of stem cells from fetal or adult brain. In contrast to this apparent homogeneity in vitro, the behavior of cells in the adult proliferative zones in vivo is more difficult to define. Nevertheless, precursor cells in the adult forebrain have been intensely studied (*19*, *54*, *56*). The proliferation of these cells can be stimulated by the direct application of mitogenic growth factors in vivo, and in animals treated in this way, proliferating cells in the subventricular zone differentiate into neurons and glia (*57*). However, in vivo less than 3% of the proliferating cells labeled with bromodeoxyuridine differentiate into neurons. The discrepancy between the efficient neuronal differentiation of adult stem cells in vitro and their inefficient differentiation in vivo is a critical but unresolved question for the field. Thus, the lack of differentiating neurons may not be a consequence of the lack of cells with the appropriate potential but rather a function of the signaling environment in the adult brain. However, a careful analysis of adult stem cells has only just

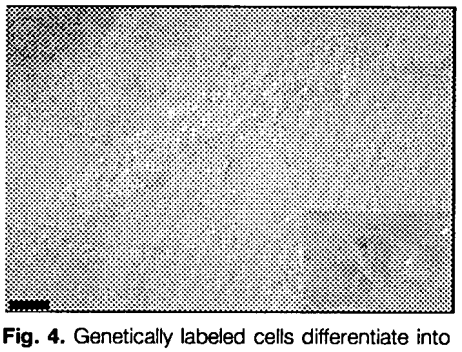

Fig. 4. Genetically labeled cells differentiate into hippocampal CA1 pyramidal neurons. The donor cells were derived from the embryonic day-14 cortical neuroepithelium of a transgenic mouse carrying a *lacZ* reporter gene. They were placed into the telencephalic vesicles of an E18 rat, where they incorporated into the host hippocampus and differentiated into granule and pyramidal neurons. The grafted cells can be identified by the blue *lacZ* signal. Data taken from (*52*). Scale bar: 50 μm.

begun, and we cannot yet rule out cell-autonomous restrictions that make the adult stem cells distinct from their fetal counterparts.

There is traditionally a close interaction between fundamental and clinical goals in the study of stem cells (58). The identification of extracellular proteins that regulate the differentiation of multipotent cells derived from the adult brain has implications for therapies targeted at neurodegenerative disease. The increased interest in extracellular signals acting on plastic cells during development fits well with the massive effort mounted in the biotechnology community to develop treatments for neurodegenerative disease based on the delivery of neurotrophic proteins. In vitro neuronal survival assays were often used in the initial identification of neurotrophic factors. These factors were then rapidly tested in animal models of neurodegenerative disease. The long-term delivery of proteins in the brain is a major goal in gene therapy. Transplantation of cells engineered to produce growth factors shows the potential of grafted cells as vectors for protein delivery (59). However, the complexity of neurotrophic signals still challenges the technology for gene manipulation and protein delivery in the CNS. There has been encouraging progress in using cell lines derived from the neuroepithelium rather than fibroblasts as cellular vectors in models of CNS disease. Neuroepithelial cells integrate in the host more readily than fibroblasts. This feature is an advantage for distributing a soluble ligand more widely in the diseased brain (60) or correcting a general biochemical deficit in the CNS (61).

It is possible to generate many different immortal cell lines from the developing CNS. These cells can express characteristics of stem cells (48, 62), neurons (63, 64), or glia (17, 65). Immortalized neuroepithelial stem cells can show extensive morphological differentiation into neurons when they are grafted into the developing (48, 62) or adult brain (64). The differentiation of genetically labeled immortal cells into neurons when implanted into the adult brain is notable because it hints that neuronal replacement in the adult is not only possible but might become simple. In most cases, immortalization has been achieved by incorporating oncogenes into a primary cell, which is, of course, not advisable for actual clinical use. However, the CRE-loxP system may be useful for removing the immortalizing oncogene before implantation (66).

More recently, the field has shifted away from the use of oncogene-immortalized cells toward the grafting of primary cells expanded in vitro. An example of this development is an experiment suggesting that primary adult cells derived from the hippocampus and cultured for long periods in vitro can still differentiate into neurons when re-implanted into the migratory pathway used to replenish neurons in the adult olfactory bulb (67). Although this field is still technically demanding, these and other results discussed here suggest that further experimental work should be directed at ambitious cell therapies based on both primary and immortal cells derived from the neuroepithelium. Clinical trials show that neuron replacement therapies for neurodegenerative diseases, such as Parkinson's and Huntington's disease, are feasible (68). Neural grafting is currently limited by a number of factors, including the lack of suitable donor material and the full integration of the grafted cells. In vitro expansion and manipulation of cells from the neuroepithelium will provide a range of well-characterized cells for transplant-based strategies for neurodegenerative disease (69). Experimental grafts in animal models suggest that the integration of grafted neurons into the circuitry of the host may be possible (50, 52, 62, 64, 69). Appropriate pretreatment of the host brain may be required for efficient neuronal differentiation by grafted precursors (70). For clinical applications, cell culture offers an important opportunity to use sophisticated genetics in cell-based therapies for neural disease.

The clinical significance of stem cell biology extends beyond cell-based therapies. The dynamics of cell organization is also critically relevant to a systematic understanding of CNS tumors and of physical injury to the brain. Two examples of nestin expression in the adult brain illustrate this point. In addition to being expressed in adult stem cells, nestin is also found in CNS tumors (71) and reactive astrocytes (72). These observations raise the interesting question of the extent of similarity between these nestin-positive cells and CNS stem cells. The proliferation and migration of CNS tumor cells are their two most damaging features. It is tempting to speculate that the self-renewing cell in a CNS tumor is similar to the stem cells found in the fetal and adult CNS.

These examples illustrate the much more general point that there will be a wide clinical impact resulting from increased knowledge of the mechanisms that control the transitions between cell types in the adult CNS. The clear-cut properties of dissociated CNS stem cells in culture show that in vitro technology can be used to define, at the cellular and molecular levels, the steps in fate choice. The presence in the adult of multipotential cells similar to the fetal stem cell emphasizes the importance of extracellular signals acting on stem cells throughout the mammalian life cycle. As our understanding of the nature of these signals grows, therapies will be developed in which the responses of normal and diseased stem cells will be manipulated to clinically useful ends.

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Marrow Stromal Cells as Stem Cells for Nonhematopoietic Tissues

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Marrow stromal cells can be isolated from other cells in marrow by their tendency to adhere to tissue culture plastic. The cells have many of the characteristics of stem cells for tissues that can roughly be defined as mesenchymal, because they can be differentiated in culture into osteoblasts, chondrocytes, adipocytes, and even myoblasts. Therefore, marrow stromal cells present an intriguing model for examining the differentiation of stem cells. Also, they have several characteristics that make them potentially useful for cell and gene therapy.

Because circulating blood cells survive for only a few days or months, hematopoietic stem cells (HSCs) in bone marrow must provide a continuous source of progenitors for red cells, platelets, monocytes, granulocytes, and lymphocytes (*1*). However, bone marrow also contains cells that meet the criteria for stem cells of nonhematopoietic tissues. The stem-like cells for nonhematopoietic tissues are currently referred to either as mesenchymal stem cells, because of their ability to differentiate into cells that can roughly be defined as mesenchymal, or as marrow stromal cells (MSCs), because they appear to arise from the complex array of supporting structures found in marrow.

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Multipotentiality of MSCs

The presence of stem cells for nonhematopoietic cells in bone marrow was first suggested by the observations of the German pathologist Cohnheim 130 years ago (*2*). Cohnheim studied wound repair by injecting an insoluble analine dye into the veins of animals and then looking for the appearance of dye-containing cells in wounds he

Neural Stem Cells for CNS Transplantation

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ABSTRACT: Neurodegenerative disorders such as Parkinson's, Alzheimer's, and Huntington's disease are becoming ever more prominent in our society. A direct approach towards therapeutic treatment of these diseases is through replacement therapy where normal tissue is transplanted back to the nervous system. Recently, significant progress has been achieved with transplants in Parkinson's disease, but the process is heavily dependent on an unstable and problematic source of fetal tissue. Neural stem cells may become the tissue/cell source necessary for developing the therapeutic potential of neural transplantation. Stem cells are self-renewing, multipotent and could provide a well-characterized and clean source of transplantable material. A number of new *in vitro* approaches have led to the development of continuously propagated stem cells that are potential candidates for nervous system transplantation. These include oncogene-induced immortalization and growth-factor stimulation of naturally occurring central and peripheral nervous system stem cells. The nature of these cells and their suitability for transplantation into the CNS will be evaluated.

Transplantation into the mammalian nervous system has long been a dream of neurobiologists. Until the 1970s only a few such experiments had been attempted and with little success. Over the last two decades a large number of rodent transplantation paradigms have been successfully employed to investigate both the survival of implanted tissue and its ability to repair damaged CNS structures.

The success of the rodent studies has led to the application of these procedures to human transplantation, particularly in Parkinson's disease. Recently some of the most promising transplantation successes have been observed in patients with MPTP-induced and idiopathic Parkinsonism.[1–3] At present, these procedures are dependent on the availability of suitable fetal mesencephalic tissue obtained from 6- to 8-week-old aborted fetuses. Currently, the state of the art technique generates fetal cells that are 70–80% viable with 10–15% of these cells immunoreactive for tyrosine hydroxylase. In the majority of cases it is estimated that only 5–10% of the engrafted tissue survives long-term at the implant site.[1]

Although these fetal tissue isolation and transplantation procedures are

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likely to improve with time, there are many obvious disadvantages for its use in brain transplantation. Serious questions arise as to whether the supply of fetal tissue will be an adequate or consistent source of safe, transplantable material for the ever increasing patient population afflicted with neurodegenerative disorders. In addition, the moral and ethical debate over the use of fetal tissue continues to be one of the most serious obstacles to this form of transplantation.

If one could choose the ideal source of cells or tissue for nervous system transplantation, it might have the following qualities: 1) the cells or tissue to be transplanted would be produced in large quantities in a reproducible fashion like a cell line; 2) the material would be well-characterized, free of adventitious agents, and would be non-transformed and non-tumorigenic when implanted into the host; 3) the transplanted tissue would be capable of differentiating into both neurons and glial cells (pluripotential), and be capable of integrating into the host brain in a relevant functional/regenerative manner, and; 4) the transplanted material would not require long-term immunosuppression for survival. All of this may be possible if homogeneous, allogenic or even xenogenic neuronal populations are transplanted which have been demonstrated to lack or express very low levels of the major histocompatibility antigens (MHC) necessary for antigen presentation.[4,5] Neural-derived stem cells possess many of the characteristics listed above as they are self-renewing and under the proper conditions capable of producing differentiated progeny,[6,7] and as such they may prove to be widely applicable for CNS transplantation.

Recently, a rodent neural cell line with a number of these characteristics has been derived from the embryonic hippocampal anlagen. These cells, termed HiB5, were derived by infection of the embryonic (E16) rat hippocampus with a retrovirus expressing a temperature-sensitive SV40 large T-antigen.[8] The cells express an intermediate filament protein called nestin[9] which is expressed *in vivo*[10] and *in vitro*[11] by neuroepithelial-derived stem cells. This intermediate filament protein appears to be specifically expressed by early stem/progenitor cells in both the peripheral and central nervous systems and upon terminal differentiation is replaced by either glial (glial fibrillary acidic protein - GFAP) or neuronal- (neurofilaments) specific intermediate filament proteins. Although the HiB5 cell line does not assume highly differentiated phenotypes *in vitro*, it can be transplanted back into the postnatally developing hippocampal dentate gyrus or cerebellum and assume differentiated morphologies and some antigenic markers associated with both neurons and glia.[8] In this respect, the HiB5 cells have been demonstrated to be multipotential after transplantation into either the developing hippocampal dentate gyrus or cerebellar granule layer. These experiments indicate that the developmental fate of at least some transplanted neural precursor cells is influenced by the CNS environment.

With respect to the ideal cell source for neural transplantation, the HiB5 line satisfies the requirement for large numbers of transplantable cells. The HiB5 line is conditionally immortalized with a temperature-sensitive SV40 T-antigen, which is inactivated at the non-permissive temperature of 37–39°C. As the rodent core temperature is believed to be in this range, the transplanted HiB5 cells appear to become non-immunoreactive for T-antigen, cease cell division, and terminally differentiate. It is reported that no tumors form anywhere in the recipient CNS after implantation of the HiB5 cells. The HiB5 cells can be transplanted allogenically into rats, but it is not clear what percentage of the cells survive and become appropriately differentiated and integrated *in vivo* and whether these cells are functionally active. These cells represent an excellent system for studying the interaction of neuronal stem cells with the normal or lesioned nervous system and satisfy many of the characteristics of a renewable source of transplantable neuronal stem-like cells. It remains to be determined how multipotenital these cells are and whether a similar version of this cell might be obtained from the human nervous system.

A number of other precursor cell lines with some of the features of the HiB5 cells have also been established from the embryonic nervous system using retrovirally mediated oncogene transfer. However, further characterization is necessary in order to demonstrate that these cells possess at least some features of true CNS stem cells.[12–15] The application of a large number of oncogenes for the derivation of continuous or immortalized neuronal and glial cell lines has yielded a vast array of these lines. Many of these immortalized cell lines have greatly contributed to our understanding of some of the complex processes and interactions within the nervous system. It is worth noting however, that the continuous nature of these lines and their capacity for producing large quantities of material is a direct result of the presence of the oncogene. Even though the expression of some of these oncogenes is conditional in nature, and some of the cell lines apparently do not readily form tumors in the rodent brain, they raise serious safety issues in the context of human transplantation.

The ideal neural stem cell would be continuous in culture, with growth and differentiation properties under normal epigenetic control and preferably not modified with transforming or immortalizing genes. In attempts to develop culture conditions for the propagation of endogenous neural stem cells *in vitro*, a number of investigators have demonstrated growth factor[11,16,17] and conditioning cell requirements[18,19] for stimulation of neuronal stem cell proliferation. However, these culture methods have not enabled the continuous propagation of cells possessing true stem cell characteristics *in vitro*. Recently, Ray *et al.*[27] have demonstrated that primary embryonic hippocampal progenitor cells can be propagated long-term in the presence of 10–20 ng/ml bFGF. This growth factor–stimulated progenitor cell population

appears restricted to the generation of nerve cells as determined by immunocytochemical and ultrastructural analysis.

Reynolds and Weiss,[20,21] have discovered a novel striatal progenitor cell that can be continuously propagated *in vitro*. The EGF-responsive progenitor cells can be isolated from embryonic[21] and adult mouse brain,[20] and can be continuously propagated in EGF-containing culture medium. Removal of EGF prevents proliferation. A single cell from one of these EGF-generated clusters can be plated by limiting dilution giving rise to an "identical" cell cluster within 10 days. Both EGF and TGFα can stimulate the propagation of similar clusters of cells while NGF, PDGF or TGFβ are not capable of stimulating cell proliferation. The majority of cells in the EGF-generated clusters also express the intermediate filament nestin. When these cell clusters are allowed to proliferate for 25 days *in vitro* (DIV), a variety of neuronal and glial phenotypes appear. These include, NSE, neurofilament, substance P, GABA, and met-enkephalin immunoreactive cells, in addition to GFAP immunoreactive astrocytes. BrdU-labeling of EGF-generated clusters between 14 and 21 DIV results in NSE and GFAP double-labeled cells at 25 DIV. These results indicate that the EGF-generated cell clusters are capable of differentiating into the majority of cell types that exist within the striatum. Furthermore, the production of differentiated BrdU-labeled neurons and glia indicate that the progenitor cells are actively dividing before terminal differentiation.

It remains to be determined whether these progenitor cells are limited to producing only cells present in the striatum or whether they are capable of generating neurons and glia found in other regions of the CNS. It is suggested from the work by Reynolds and Weiss that the EGF-responsive cells have self-renewing properties in that isolated cell clusters can be split into single cells which can reproducibly give rise to a multipotential cluster of cells containing an unknown number of the original "stem" cells. It is suggested that these stem-like cells can continue to be self-renewing and divide in an asymmetric fashion (divide to produce an identical stem cell and a more differentiated daughter cell) and are capable of giving rise to neuronal and glial cells throughout the life span of the organism. In support of this idea, apparently identical, multipotent EGF-dependent cells can be isolated from both E14 and adult mouse brain. Currently, these cells remain pluripotential up to 30 passages *in vitro*. It is very likely that the true potential of these cells will be discovered using a combination of *in vitro* (growth factor, co-culture) and in vivo differentiation paradigms similar to those used for the HiB5 cells.

It will also be important to relate the Weiss/Reynolds EGF-dependent progenitor cells to the O_2A precursor cell which gives rise to the oligodendrocyte and type II astrocyte described by Raff and collaborators[22,23] and to the recently identified NO precursor cell[24] that gives rise to both neurons and oligodendrocytes.

Interestingly, it has been previously reported that EGF and TGFα also stimulate retinal neuroepithelial cell mitosis in primary cultures.[25] This work clearly shows that a variety of retinal neuronal and glial phenotypes can be generated in these cultures in the presence of EGF and low serum conditions. Under these culture conditions these cells were not demonstrated to be capable of continuous propagation.

Finally, recently published experiments indicate that a multipotent neural crest stem cell can be identified and cultured *in vitro*.[26] It is of interest to mention here that the initial culturing medium contains 100 ng/ml EGF together with βFGF and 2.5S NGF. The neural crest stem/progenitor cell can be isolated by FACS sorting of surface labeled crest cells or through replating and cell surface labeling using antibodies to low affinity nerve growth factor receptor (LNGFR). In this work, Stemple and Anderson have demonstrated that all of the LNGFR positive neural crest cell progenitors express high levels of the intermediate filament protein, nestin. Primary clones cultured for 9–14 days generated neuronal morphologies reacting with antibodies to neurofilament 160KD, N-CAM, and peripherin. Peripherin is an intermediate filament protein expressed almost exclusively within the peripheral nervous system. These differentiated cells no longer express nestin or LNGFR. Furthermore, addition of forskolin (5μm) and 10% FBS to the culture medium results in the expression of Schwann cell markers, GFAP, P_0 and sulfatide-0_4 in many of the non-neuronal cells remaining in these cultures.

If 6 day-old LNGFR receptor positive clones are dispersed into single cells and replated, 50% of those cells survive and grow into colonies that produce peripherin positive neurons and GFAP-positive Schwann cells. These experiments suggest that the neural crest progenitor/stem cell is multipotent and can reproduce both asymmetrically and symmetrically giving rise to itself and a differentiated daughter cell.

Although the neuronal phenotype(s) produced by these cells are not known, it is clear that both neurons and Schwann cells can be generated from a single neural crest "stem" cell and that these cells appear to be both self-renewing and pluripotential. It now remains to be demonstrated whether these cells can be continuously propagated and employed for *in vivo* transplantation paradigms.

In summary, a number of neuroepithelial derived "stem" cell populations have been adapted to the culture environment. This is a significant advance towards developing a reproducible and highly manipulatable source of uniform cells for application to transplantation in the nervous system. Whether these cells can be continuously cultured with no loss of renewing potential remains to be shown. Furthermore, it is not clear whether any of the cells described are pluripotential for all CNS and PNS cell types or have a more limited potential. Finally, there is little evidence for the ability of these "stem"-like cells to be successfully transplanted into the nervous system. Much work

remains to determine the true potential of these putative nervous system stem cell populations, but the prospects are incredibly exciting.

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ALZHEIMER'S DISEASE
AMYLOID PRECURSOR PROTEINS, SIGNAL TRANSDUCTION, AND NEURONAL TRANSPLANTATION

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[17] Transplantation of Epidermal Growth Factor-Responsive Neural Stem Cell Progeny into the Murine Central Nervous System

Joseph P. Hammang, Brent A. Reynolds, Samuel Weiss, Albee Messing, and Ian D. Duncan

Introduction

Neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's disease, as well as demyelinating disorders such as multiple sclerosis (MS), are of serious concern in our society. Our understanding of the processes underlying these disorders is poor and treatments are few. Because of the wide array of central nervous system (CNS) disorders, possible therapeutic approaches are also diverse and include cell replacement via transplantation; neurotrophic factor delivery from implants of polymer-encapsulated or unencapsulated, genetically modified cell lines; and the systemic delivery of small, therapeutic molecules capable of traversing the blood–brain barrier. As each of these approaches has benefits and limitations, it may be necessary to combine some of them for successful therapeutic intervention.

Much attention is currently being directed toward the use of neurotrophic factors in the treatment of neurodegenerative disorders. The application of these factors to the CNS for therapeutic intervention has been delayed primarily by two obstacles. First, polypeptide growth factors are relatively large molecules and, in general, are unable to cross the blood–brain barrier. Second, it is unclear whether non-site-specific delivery will be effective in reversing the degenerative process that typically occurs in defined brain regions and whether nonspecific delivery will produce detrimental side effects. Although specific small molecules, capable of crossing the blood–brain barrier may be developed to overcome some of these problems, few options exist today for the delivery of neurotrophic factors to the CNS. One option is the use of a polymer encapsulation technology that provides a means for the delivery of neurotrophic factors and neurotransmitters to the site of disease within the nervous system (see Chapters [22]–[24], this volume). With this technology, one can safely encapsulate cell lines that have been genetically modified to secrete neurotrophic or other factors. The encapsu-

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lated device provides protection to the host from uncontrolled cell line growth while protecting the implanted cells from the host immune system.

A second approach in the treatment of neurodegenerative diseases is through cell replacement, by which unprotected cells are transplanted into the damaged or diseased nervous system. Potential cell therapy applications in humans include the replacement of oligodendrocytes in demyelinated MS lesions, replacement of dopaminergic neurons of the substantia nigra in Parkinson's disease, or the site-specific implantation of genetically modified, trophic factor-secreting cells to prevent or arrest neuronal loss. In experimental approaches for the treatment of demyelinating and inherited dysmyelinating disorders, progress has been made in animal models with the implantation of dissociated primary glial cells as well as a number of oligodendrocyte precursor cell lines. In these paradigms, oligodendrocytes or oligodendrocyte precursors have been shown to restore myelin in portions of the demyelinated or dysmyelinated nervous system (1–3). Progress has also been achieved with fetal cell transplants into patients with Parkinson's disease; however, the procedure has been largely dependent on the availability of fetal tissue, which is fraught with concerns of a moral and ethical nature as well as concerns over the uniformity, adequate supply, and the safety of the donor material (4, 5).

The lack of a reliable and safe supply of primary cells for human transplantation has resulted in the development of cell lines, specifically the oncogene-induced immortalization of CNS stem/progenitor cells (for reviews see Refs. 6 and 7). Although these cells have been extremely valuable in rodent transplant models, and in broadening our understanding of neural development and function, the use of oncogene-driven cells for human transplant therapy is questionable. Although the use of cell lines derived with temperature-sensitive or inducible oncogenes may be a logical alternative, their safety for transplantation into humans has not been addressed. An epidermal growth factor (EGF)-responsive neural stem cell culture system has been developed that may be an alternative to oncogene-generated cells (8–10). These growth factor-responsive, nontransformed neural stem cells can be continuously propagated in a cell line-like manner for indefinite periods in the presence of EGF. On removal of EGF, proliferation stops and the stem cell-generated progenitor cells can be differentiated *in vitro* into neurons, astrocytes, and oligodendrocytes.

In this chapter we describe aspects of this novel EGF-responsive stem cell culture system. The EGF-responsive cells can be isolated from embryonic and adult rat and mouse brain, and similar cells have been isolated from fetal human brain (B. Reynolds *et al.*, unpublished observations, 1994). In addition, we demonstrate that undifferentiated stem cell progeny are capable of forming oligodendrocytes when transplanted *in vivo*. Finally, we describe the development of genetically tagged, EGF-responsive stem cells derived

from transgenic mice. These mice carry chimeric genes composed of mammalian cell-specific promoter elements that direct the expression of a reporter gene to either astrocytes or oligodendrocytes.

Production and *in Vitro* Characterization of Epidermal Growth Factor-Responsive Neural Stem Cells

Using sterile technique, the striata from litters of E14–15 Sprague-Dawley rats, or of BALB/cJ or CD1 mice, are dissected and separately pooled in L-15 dissection buffer (GIBCO, Grand Island, NY) and held on ice. The L-15 is removed and the dissected tissue is resuspended in a defined, DMEM: F12-based (GIBCO) serum-free medium containing glucose (0.6%), insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 n*M*), putrescine (60 μM), selenium chloride (30 n*M*), glucose (0.6%), glutamine (2 m*M*), sodium bicarbonate (3 m*M*), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (5 m*M*) (all reagents from Sigma, St. Louis, MO), and EGF (20 ng/ml; Collaborative Research Incorporated, Waltham, MA) (subsequently referred to as complete EGF medium). The tissue is vigorously triturated 10–20 times with a fire-polished Pasteur pipette to achieve a single-cell suspension and the dissociated cells are plated at 100,000 cells/ml in T25 flasks and maintained in the complete EGF medium. Over the following 7–10 days, free-floating spheres of proliferating cells are formed. Without disturbing the attached cells, the spheres are removed, gently centrifuged at 800 rpm, and triturated with a fire-polished pipette to a single-cell suspension and plated at approximately 100,000 cells/ml in T25 flasks. By this method, the cells can be subcultured once per week (i.e., passaged) repeatedly, forming nonadherent spheres that float in suspension. The progeny can be identified by their immunoreactivity with antiserum to nestin, an intermediate filament protein expressed by undifferentiated neural stem/progenitor cells (11, 12). Under the appropriate differentiating conditions, the progeny of the EGF-generated cells lose their nestin immunoreactivity and differentiate into neurons, astrocytes, and oligodendrocytes. These EGF-responsive stem cells have now been routinely passaged more than 50 times over a period of 1 year and remain nestin immunopositive and multipotent.

Identification of Stem Cell Progeny and Their Differentiation Potential *in Vitro*

Differentiation and Immunolabeling Procedure

The EGF-generated cells can be induced to differentiate into oligodendrocytes, astrocytes, and neurons by altering the culture conditions (see Fig.

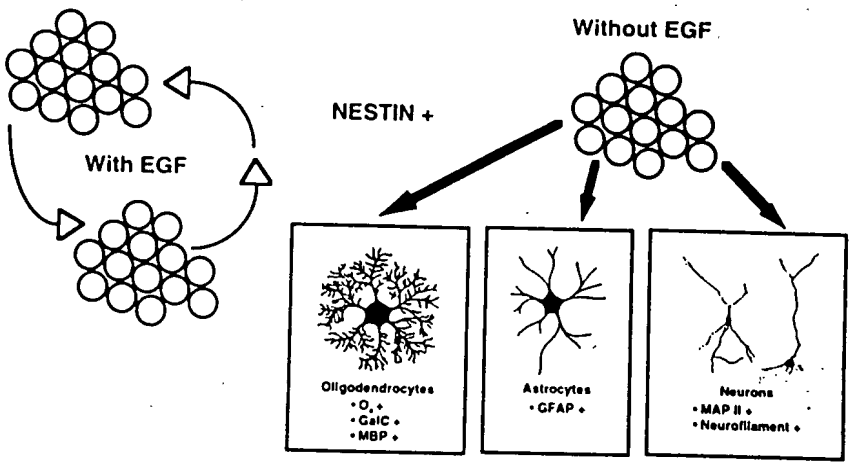


FIG. 1 Schematic representation of the EGF-responsive stem cell culture system. In the presence of EGF, and in a defined, serum-free medium, the stem cells can be propagated indefinitely. The stem cells grow as spheres (neurospheres) that are nestin positive. The stem cells can be influenced to differentiate by removing the EGF, and adding a small amount of serum to the defined medium. Under these conditions, the cells rapidly form the three major cell types in the CNS: neurons, astrocytes, and oligodendrocytes. The relative proportion of each differentiated cell type can be influenced using combinations of growth factors.

1). The free-floating, EGF-generated spheres are gently centrifuged, resuspended in the base medium (minus EGF) with 1% (v/v) fetal bovine serum, and plated on poly-L-ornithine-treated glass coverslips (10 μg/ml). The EGF-generated spheres attach firmly to the glass, and the cells slow or stop dividing and begin to differentiate. One to 14 days postplating, the cells on the coverslips are incubated unfixed, for 30 min at room temperature, with one of the following primary monoclonal antibodies; O1, O4 (13), galactocerebroside (GalC), or A2B5 (supernatants) (all provided by P. Wood, University of Miami) diluted in minimal essential medium with 5% (v/v) normal goat serum and 25 m*M* HEPES buffer, pH 7.3 (MEM–HEPES, NGS). Coverslips are gently washed five times in MEM–HEPES, and incubated for 30 min at room temperature in fluorescein- or rhodamine-conjugated secondary antibodies (Sigma) diluted in MEM–HEPES, NGS as recommended by the manufacturer. The coverslips are then washed five times in MEM–HEPES and fixed with acid alcohol (5% glacial acetic acid–95% ethanol) for 30 min to 1 hr at −20°C. Following this fixation, the coverslips are washed five times with MEM–HEPES, and either mounted and examined using fluorescence microscopy or immunoreacted with rabbit polyclonal antisera raised against glial fibrillary acidic protein (GFAP) (Dako, Carpinteria, CA), nestin (R. McKay, NIH), myelin basic protein (MBP) (Dako), or proteolipid protein

(PLP) (Serotec, Serotec Products, Harlan Bioproducts for Science, Inc., Indianapolis, IN). When subjected to a second round of immunolabeling, the coverslips are incubated first for 1 hr with 5% NGS in 0.1 *M* phosphate buffer with 0.9% (w/v) NaCl at pH 7.4 (PBS) followed by rabbit primary antibodies diluted in NGS for 1–2 hr at room temperature. Coverslips are washed three times with PBS, incubated with the appropriate secondary antibody conjugates diluted in NGS, washed with PBS, and then mounted on glass microscope slides with antifadent (Citifluor, Ltd., London, UK) mounting medium and examined using a fluorescence microscope. In cases in which samples are immunoreacted with antibodies raised against intracellular antigens and not immunolabeled live with the monoclonal antibody supernatants, the coverslips are fixed for 20 min with 4% paraformaldehyde in PBS (pH 7.4), washed with PBS, permeabilized with 100% ethanol, washed again with PBS, and incubated with 5% NGS in PBS for 1 hr. Primary antibodies and secondary antibody conjugates are applied as outlined above.

Formation of Mature Oligodendrocytes in Vitro from Epidermal Growth Factor-Responsive Stem Cell Progeny

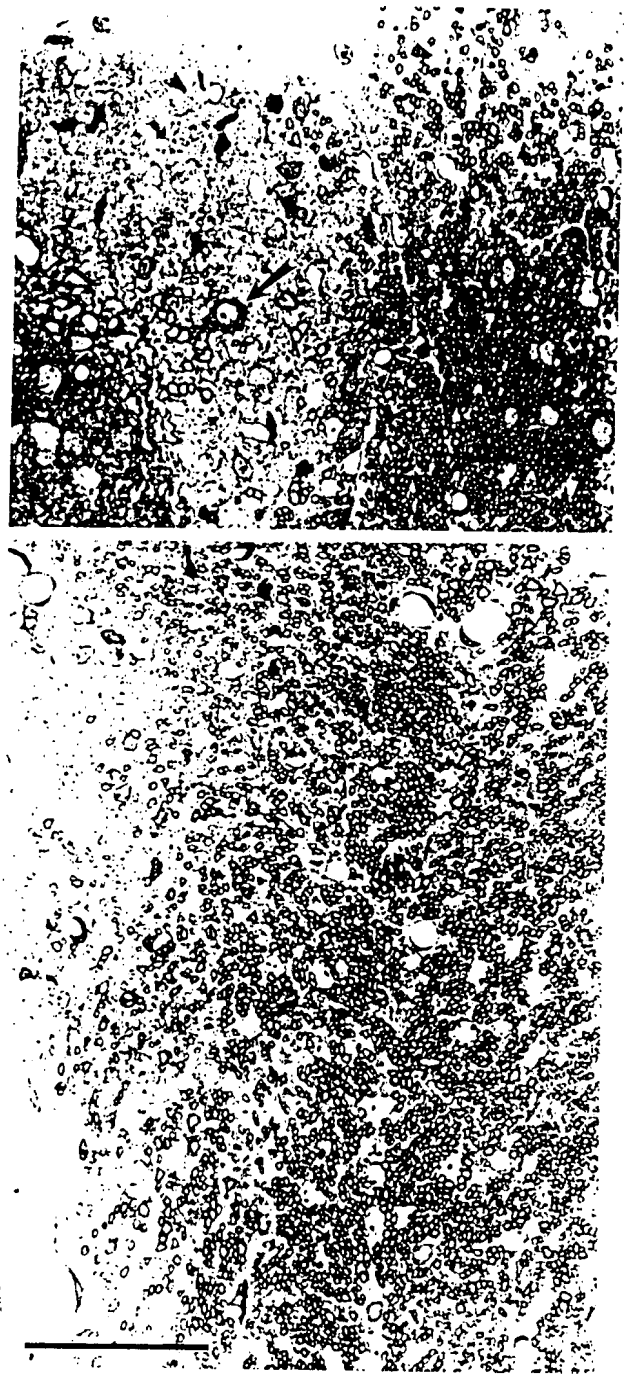
To identify specific cell types 1 to 7 days after differentiation, cells on coverslips were immunolabeled with antibodies specific for oligodendrocyte precursors or mature oligodendrocytes. At 1 day postplating, the spheres attach to the substrate and many cells with a bipolar morphology migrate from the sphere. Most of the cells, especially flat cells at the base of the cluster, are immunoreactive for nestin. By 3 days, however, the nestin immunoreactivity in these flat cells diminishes whereas GFAP immunolabeling increases, consistent with the differentiation of astrocytes. During this same 3-day period, the bipolar cells, which are initially O4 positive, exhibit a decrease in O4 immunoreactivity and a concomitant increase in GalC immunolabeling by the third day postplating. Five to 7 days postplating, nestin and O4 immunoreactivity is significantly reduced in all cells and a fraction of the $O4^+/GalC^+$ oligodendrocyte precursors become immunoreactive to O1, MBP, or PLP. At this stage of differentiation in the culture system, these cells also possess a distinct oligodendrocyte morphology.

Formation of Myelinating Oligodendrocytes by Epidermal Growth Factor-Responsive Stem Cell Progeny when Transplanted *in Vivo*

Because the EGF-responsive stem cells are capable of generating oligodendrocyte precursors and oligodendrocytes *in vitro*, we examined whether these cells could respond to natural cues by differentiating into oligodendro-

cytes and myelinating CNS axons *in vivo*. The myelin-deficient rat (*md*) is an inherited dysmyelinating mutant and during their brief life span (approximately 25 days) the affected animals form virtually no myelin within the CNS. Prior to transplantation into the *md* rat, the EGF-responsive stem cells are maintained in EGF-containing medium for up to 35 passages. Nestin-positive cells (no mature oligodendrocytes) are collected and triturated into a single-cell suspension in the presence of 0.1% (w/v) bovine serum albumin (BSA). Epidermal growth factor-generated cells from different passages are used for injection into the mutant spinal cords. Myelin deficiency is an X-linked recessive trait and therefore only one-half of the male offspring born to carrier females are affected hemizygotes for the mutation. After laminectomy and exposure of the spinal cords, the mutants are readily identified at postnatal days 8–10 by the absence of myelin within the dorsal columns. At postnatal days 8–10, animals are anesthetized with halothane, a small longitudinal incision is made along the back, and a laminectomy performed, exposing the T13–L1 level of the spinal cord. A small incision is made in the dura mater to allow for the entry of the glass micropipette. To limit the number of animals, only the mutants are used for injection. Approximately 1.0–1.5 μl (50,000 cells/μl) of the stem cell progeny in Hanks' balanced salt solution (HBSS) is then injected into the dorsal columns just lateral to the midline. The site of injection is marked using sterile charcoal powder. Following the injection, the incision is sutured and the animals are gently warmed and allowed to recover from the halothane anesthesia. The animals are then returned to the dams and allowed to survive for approximately 2 weeks. Because the mutants usually die by approximately postnatal day 25, they are sacrificed at 21–24 days of age, using a pentobarbital overdose and aldehyde perfusion. Rats transplanted with the mouse stem cell progeny receive cyclosporin A (Sandoz, Switzerland) at a dose of 10 mg/kg intraperitoneal (ip), commencing on the day prior to cell injections. Spinal cords are removed and further postfixed in the same aldehyde fixatives for at least 24 hr, postfixed with osmium tetroxide, dehydrated, and processed for Epon embedding (1).

FIG. 2 The undifferentiated stem cells are capable of differentiating into myelinating oligodendrocytes when implanted into the myelin-deficient CNS. Photomicrographs of a section of the dorsal columns of an *md* rat spinal cord (T13–L1) 2 weeks postimplantation of undifferentiated mouse neurospheres. There is abundant normal myelin, especially along the midline in these sections. *Top:* At the edge of the photomicrograph, an apparent oligodendrocyte is seen that is in close contact to a cluster of myelinated fibers (arrow). One-micrometer Epon-embedded section stained with toluidine blue. Bar: 100 μm.



Histological analysis on toluidine blue-stained, 1-μm Epon sections was performed on the implanted spinal cords. Varying numbers of myelinating oligodendrocytes were seen in the implanted spinal cords with all myelin being confined to the dorsal columns (Fig. 2). Generally, within the core of the myelinated zones, the myelinated fibers were densely packed, and the myelin appeared to be of normal thickness, although this was not quantitated. At the edges of the zones, a number of myelinating oligodendrocytes were seen in contact with several axons. There was no obvious evidence of inflammation or abnormal cell death in any of the implant sites by light or electron microscopy.

In vitro, the majority of the EGF-responsive stem cell progeny form astrocytes under the standard differentiation protocol. Therefore, it was possible that astrogliosis would have been seen in the injected spinal cords. To determine whether the stem cell-derived astrocytes directly or indirectly caused any astrogliosis, we examined regions of the spinal cords that had received stem cell implants using immunocytochemistry for GFAP. For this analysis, we used 1-μm Epon sections that were essentially adjacent to those used in the analysis of the extent of myelination. None of the spinal cord sections containing the implanted stem cell progeny exhibited an increase in GFAP immunoreactivity relative to the spinal cords in the uninjected *md* rat. Furthermore, electron microscopy (EM) analysis of the grafted areas revealed no evidence of hypertrophied astrocyte fibers. Together, these experiments indicate that the injection of the stem cell progeny does not lead to glial scarring and that the implanted cells preferentially differentiate into oligodendrocytes in resopnse to the nonmyelinated CNS.

Although we have demonstrated a significant amount of myelin formation in the mutants, their short life span may limit the extent of myelination that is possible during this period. Because of the short interval between cell injection and sacrifice it is possible that some cells retained the potential to divide and myelinate at a later time. To address this issue, we performed [^{3}H]thymidine autoradiography on semithin sections to determine the extent of cell division in the injected and the uninjected mutants in the regions of myelination. [^{3}H]Thymidine labeling was seen within the myelinated patches in all animals examined. This is consistent with the development of stable, mature oligodendrocytes within the core of myelinated fibers, whereas in regions containing naked axons a few precursor cells remain with the capacity to divide. It is important to note that we observed no hyperplasia or hypercellularity as a result of the injections in any of the animals examined in this study. Although the number of labeled cells seen at the periphery of the myelinated zones in the injected animals appears to be significantly greater than that seen in the naive *md* rats, quantitation of labeling indices is required.

Differentiation and Survival of Epidermal Growth Factor-Responsive Stem Cells, Genetically Tagged with *Escherichia coli* β-Galactosidase Gene, When Implanted into Mouse Cerebral Cortex

We were interested in determining if the genetically tagged stem cell progeny would integrate into the developing CNS and if they would continue to express the reporter gene *in vivo*. Reports have demonstrated the negative regulation of retroviral long terminal repeats (LTRs) in transplanted cells, possibly through the actions of cytokines within the CNS (14). Proliferating,

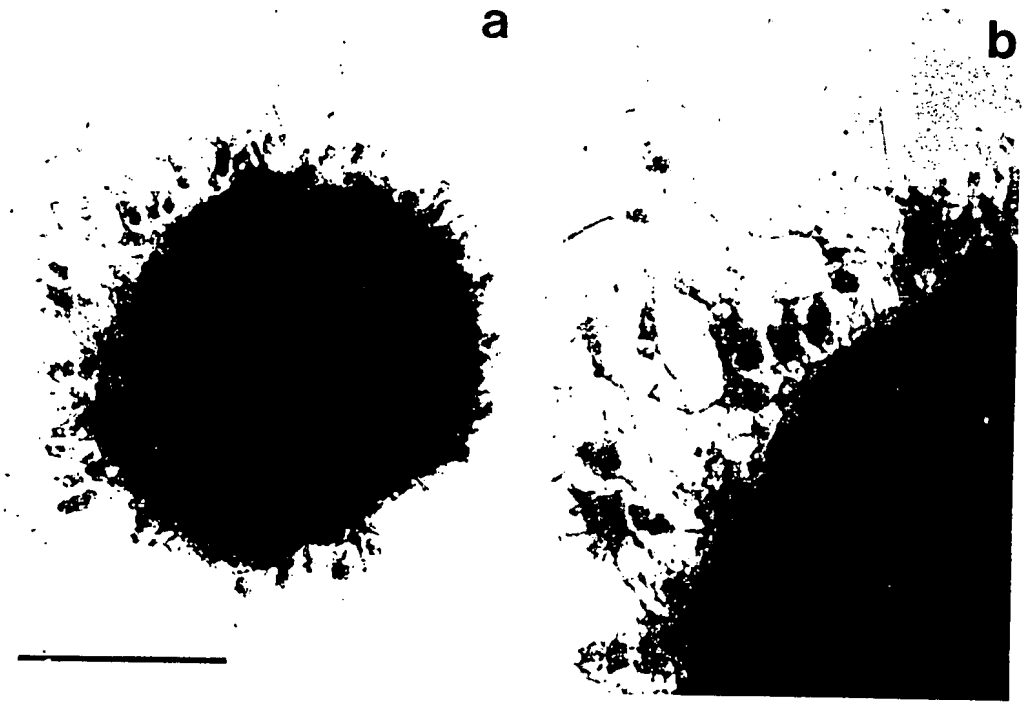


FIG. 3 Genetically modified EGF-responsive stem cells express the β-galactosidase reporter gene *in vitro*. A photomicrograph of genetically modified stem/precursor cells infected with a retrovirus containing the β-galactosidase gene (a). Cells containing the gene exhibit a characteristic blue reaction product after X-Gal histochemistry. (b) A higher magnification photomicrograph of (a), showing labeled cells migrating away from the sphere, extending processes, and taking on the morphology of differentiated CNS cells. Bar: 50 μm.

EGF-responsive cells are exposed to conditioned medium from the CRE BAG 2 packaging cell line (ATCC CRL 1858) for 1 day in the presence of Polybrene (8 μg/ml). Following this exposure, the cells are selected in Geneticin (400 μg/ml) (G418; GIBCO). Antibiotic-resistant spheres form within the first week and some of these spheres are expanded for several passages to produce sufficient cells for implantation (Fig. 3). One- to 3-day-old CD1 mice are anesthetized on ice. A small incision is made in the skin, a flap of bone is retracted, and a 2- to 3-mm^2 area of cortex is removed by aspiration. Undifferentiated mouse stem cell progeny are prepared for injection as described in the previous section. Approximately 50,000 cells are injected into each animal, using a micropipette. Three to 4 weeks later, the animals are deeply anesthetized with pentobarbital and perfused with 4% paraformaldehyde. The brains are removed, postfixed overnight in the same fixative, and cryoprotected in 25% sucrose and frozen in liquid nitrogen. The brains are cryosectioned (10–15 μm) and every fifth section is taken through the implantation region and processed for β-galactosidase activity, using the method of Vandaele *et al.* (15) with minor modifications. β-Galactosidase-labeled cells are seen in all of the animals (Fig. 4). These results demonstrate that transgenes inserted *in vitro* can be expressed *in vivo* following transplantation of the genetically modified, EGF-responsive stem cells.

Transgenic Mouse-Derived Neural Stem Cells: A Source of Marked Glial Cells for Central Nervous System Transplantation

In the section Formation of Myelinating Oligodendrocytes (above), we described the implantation of the EGF-responsive stem cell progeny into the dysmyelinated CNS. In this transplant paradigm, the presence of abundant myelin at the site of the implant clearly identified cells of donor origin. However, for the majority of transplant studies, the identification of donor cells or tissues after implantation remains a significant challenge. In some cases it is possible to identify cells of xenogeneic origin within the host using species-specific antibodies such as M6 (16, 17). Current strategies used to tag donor cells prior to implantation suffer from inherent problems of dilution, toxicity, and stability of gene expression over the long term. Furthermore, methods used to tag cells genetically *in vitro* with reporter genes generally suffer from low efficiency and the eventual identification of the cells *in vivo* is made more difficult if a majority of the implanted cells are never labeled in culture.

To provide a more stable and efficient method of cell labeling, we have used the promoter elements for the human glial fibrillary acidic protein (GFAP) gene (18) and the human myelin basic protein (MBP) gene (L. Wra-

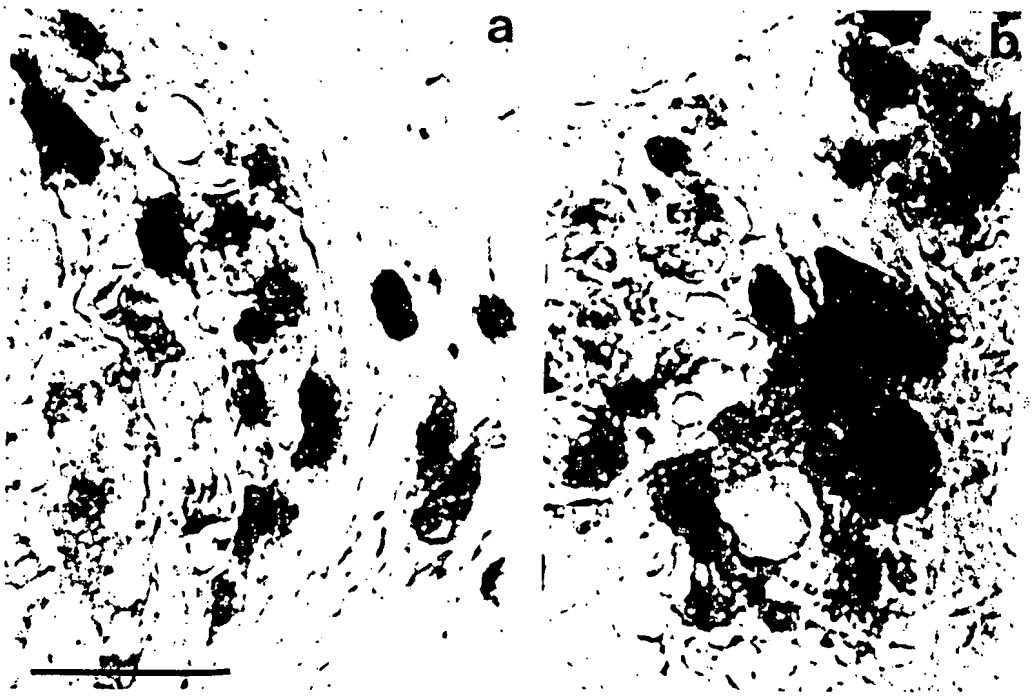


FIG. 4 EGF-responsive stem cells, genetically modified *in vitro*, survive transplantation. Photomicrographs of a section of cerebral cortex from a 4-week-old mouse that had been implanted with genetically modified cells on the day of birth (a and b). X-Galactosidase histochemistry was performed on free-floating, Vibratome sections. The large number of blue cells demonstrates that the genetically modified stem cell progeny can survive transplantation and express the transgene. Bar: 50 μm.

betz, personal communication) to direct the expression of the *Escherichia coli* β-galactosidase reporter gene in transgenic mice. Transgenic mice are produced using standard pronuclear injection of the MBP and GFAP constructs into fertilized F2 mouse eggs derived by crossing B6SJLF1 parents. Transgenic lines are maintained by back-crossing to B6SJLF1 mice. Epidermal growth factor-responsive stem cells have been prepared from individual fetuses from both of these transgenic mouse lines and propagated in the presence of EGF in serum-free, defined medium. *In vitro*, the stem cells derived from these transgenic animals appear to be identical to those derived from nontransgenic animals in their ability to proliferate and differentiate. When the stem cell progeny are allowed to differentiate, cell-specific expression of the reporter gene occurs in astrocytes (GFAP-*lacZ*) and in oligoden-

drocytes (MBP-*lacZ*) in a developmentally regulated manner. Identification of the β-galactosidase-positive cells is accomplished using double labeling with antibodies raised against GFAP (Dako) or MBP (Dako). The expression of the reporter genes is highly stable, as it is seen in thirtieth passage cultures and appears to be expressed in virtually 100% of the appropriate cells. We are currently investigating the expression of the reporter gene after transplantation in the rodent CNS. The use of mammalian promoter elements that are cell specific should eliminate the problems of inactivation or negative regulation seen with the use of retroviral LTRs. Transgenic mouse-derived neural stem cells represent a novel means of obtaining genetically tagged, stable populations of cells for transplantation.

Conclusions

Central nervous system stem cells represent a novel source of cell types for future transplant therapies, both for neuronal or glial cell replacement. Although the experiments that are presented here are of a preliminary nature, stem cells derived from humans could prove to be widely applicable in CNS transplantation. Studies are in progress to examine the feasibility of inducing differentiation of the stem cell progeny in culture prior to transplantation in the CNS in order to produce neural cells with specific phenotypes.

Acknowledgments

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Providing Pharmacological Access to the Brain: Alternate Approaches

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MOLECULAR BRAIN RESEARCH

Short communication

Non-virally mediated gene transfer into human central nervous system precursor cells

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Abstract

Lipofectamine-based transfection was used as a method of choice to deliver the bacterial β-galactosidase gene into human central nervous system (CNS) precursor cells. We achieved a transfection efficiency of 7.4%. β-Galactosidase expressing cells were shown to display both neuronal and glial phenotypes. We also delivered the temperature sensitive allele of SV40 Large-T antigen and obtained a high level of expression of the immortalizing oncoprotein in the cells. Colonies of Large-T antigen immunoreactive cells were indeed visible 10 days after transfection.

Keywords: Human CNS precursor cell; Transfection; Lipofectamine; Large-T antigen

During the development of the central nervous system (CNS), multipotent precursor cells rapidly proliferate to give rise to transiently dividing progenitors that will eventually differentiate into the several cell types that compose the adult brain [18]. These precursors have been isolated from the embryonic CNS of several mammalian species and shown to retain properties of immature cells such as Nestin expression [16], extended proliferative potential and the capacity to give rise to differentiated progeny [9,25]. The factors and genes that regulate CNS precursor cell proliferation and progenitor differentiation have begun to be investigated thanks to the identification of cell culture methodologies enabling specific populations of immature cells to be maintained in vitro [3,4,10,14,20,23,26] and to advances in gene transfer techniques that allow the introduction of genes of interest into these cells [2].

Introduction of foreign genes into human CNS cells has only recently been attempted. In a recent report the *LacZ* gene was vehicled to human brain progenitors via adenoviral vectors [24]. However, the possibility to genetically modify proliferating human CNS precursor cells would be of fundamental importance for improving our knowledge of events that occur in the human brain under normal and pathological conditions. Among the different methodologies available to deliver foreign genes into primary rodent cells, retroviral vectors have been utilized most widely [6]. While the use of ecotropic host viruses to transduce genes into rodent cells presents no major safety risks, the need for amphotropic retroviruses to target human cells [7] generates limitations to their application due to the great biohazard that these vectors present. Strategies for adenovirus (Ad), adeno-associated virus (AAV) and herpes virus (HSV) mediated gene transfer have also been developed [8,15]. Recombinant Ad as well as HSV have indeed been utilized in gene therapy due to their high efficiency of infection and reduced safety risks. Neverthless they rarely integrate into host genome as they remain a non-replicating extrachromosomal entity [8,15]. Although AAVs have some attractive advantages (for example a preferred site of integration into the human genome) the limited knowledge on expression and function of AAV genes makes these vectors not yet widely employed [15].

With the purpose of identifying efficient gene transfer approaches that are suitable for human CNS precursor cells and that avoid the use of viral vectors, we have used the Lipofectamine-based methodology and tested its efficiency in delivering the bacterial β-galactosidase (*LacZ*) gene. Recently, transfection methodologies that utilize a

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similar lipid-based reagent have been successfully applied to rodent primary cultures of hippocampal neurons, allowing to obtain a transfection efficency of approximately 3% [13].

Multipotential human fetal CNS precursor cells were previously obtained from the diencephalon of a 10.5-week-old fetus [27]. These cells have been serially subcultured and expanded for longer than 1 year in serum-free growth medium containing epidermal growth factor (EGF) and shown to have a duplication time of approximately 30 days (A.V., in preparation). No changes in their growth characteristics or differentiation potential (see below) were observed over this period (A.V., not shown). Human CNS precursors, as well as their rodent homologues, maintained in EGF-containing medium, grow in suspension forming aggregates of variable sizes named "neurospheres" [23,26,27]. It has been observed that upon removal of the growth factor, these cells can differentiate into neurons, expressing multiple neuronal antigens, as well as glia [23,26,27].

The day before transfection the human "neurospheres" were collected and dissociated using trypsine 0.5 × in Ca^{2+}/Mg^{2+}-free HBSS for 10 min at 37°C. Dissociated cells were plated onto laminin-coated (20 μg/ml) 24-well dishes. Transfection was performed using the Lipofectamine method (Gibco-BRL, Life Technologies, Italy). We achieved the best working conditions by varying several parameters. In particular we varied the density of cells seeded, the amount of DNA and Lipofectamine utilized, and the time of exposure to the reagent (shown below). The day after transfection cells were rinsed 3 times with PBS (8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4) and fresh growth medium was added. To identify the best transfection conditions we utilized the CMV-β plasmid carrying the *LacZ E. coli* gene under the control of the CMV promoter; plasmid DNA was prepared using cesium chloride purification. Results shown in Fig. 1 indicate that the optimal parameters to obtain the highest percentage of histochemically reacted X-Gal positive human CNS cells are 2×10^5 cells/cm^2, 1 μg of DNA, 2% Lipofectamine and 5 h of exposure to the reagent. Under these conditions the efficiency of transfection (number of blue cells over number of plated cells) reached 7.4% (Fig. 1A and Fig. 2A). When different amounts of plasmid DNA or Lipofectamine were used (Fig. 1A) or when exposure times to the transfectant or number of cells seeded were varied (Fig. 1B), a decreased efficiency of transfection was observed. In particular conditions (asterisks in Fig. 1) a reduced cell viability was observed as evaluated by Trypan blue exclusion. Furthermore, we found that concentrations of Lipofectamine over 2% were toxic to the cells.

This transfection procedure did not alter the ability of the cells to differentiate. Following transfection, the cultures were incubated for 6 days in serum-free medium deprived of growth factors, and thereafter cells were fixed with 4% paraformaldehyde. Transfected cultures were

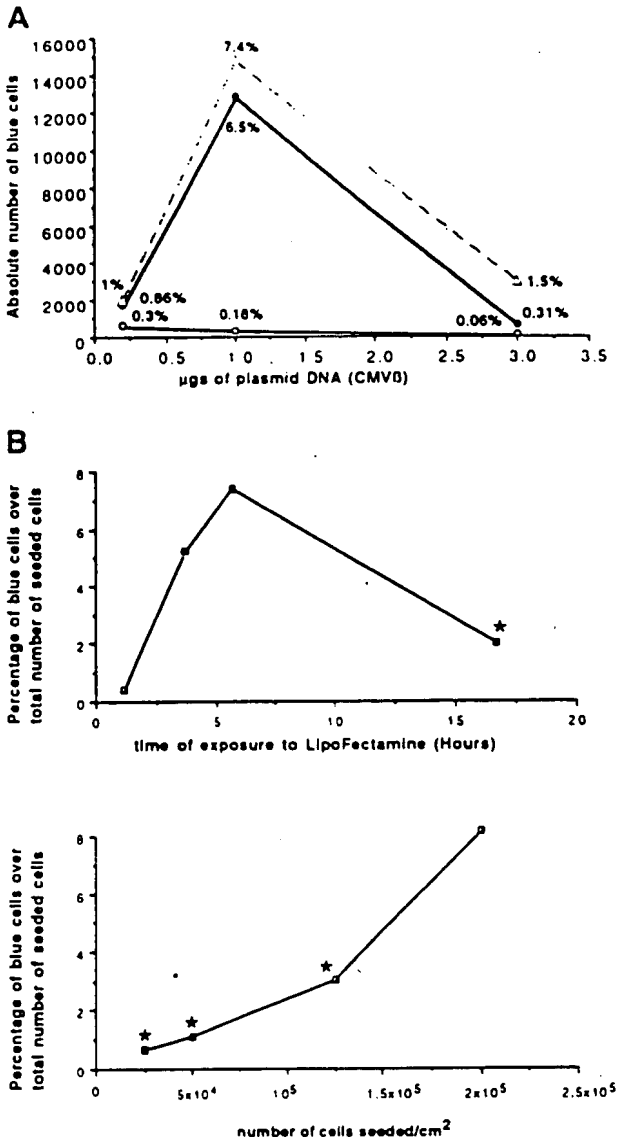


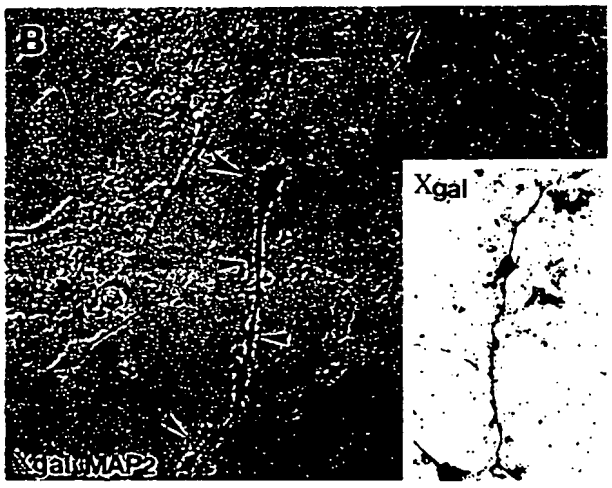
Fig. 1. Efficiency of transfection into human CNS precursor cells. In A, results are provided as absolute number of blue cells out of 2×10^5 cells/cm^2 which have been exposed to Lipofectamine for 5 h. Cells were transfected with increasing amount of plasmid DNA in the presence of 0.3% (○), 1% (●) and 2% (△) Lipofectamine. Percentage of blue cells in each condition is also indicated. The number of transfected cells was estimated by counting all blue cells present in a 2 cm^2 dish. Duplicate dishes were counted per each condition. In B, the number of transfected cells (expressed as percentage) is evaluated after different exposure times to the reagent or under various plating densities. In B cells were transfected with 1 μg of plasmid DNA in the presence of 2% Lipofectamine. Other combinations of DNA and Lipofectamine were less effective. Asterisks in the graphs indicate the conditions in which cell viability was reduced (over 30% of dead cells as judged by Trypan blue exclusion). Data shown are from one out of three experiments that provided similar results.

therefore double-stained for β-galactosidase and for the neuron-specific microtubule-associated protein MAP2 or the glia-specific intermediate filament GFAP. For the identification of the phenotype of the transfected cells, the

combination of the *LacZ* histochemical reaction with the immunodetection of MAP2 or GFAP was also utilized. As shown in Fig. 2B, cells expressing β-galactosidase are positive for MAP2. In the inset a transfected bipolar cell is visible. In Fig. 2C β-galactosidase positive cells (left panel) are immunolabeled with anti-GFAP antibodies (right panel). As shown, the majority of the cells in the figure are double labeled with the two antibodies. Examples of a transfected cell that fails to express GFAP (arrow) and of two GFAP positive untransfected cells (arrowhead) are also visible providing evidence on the specificity of the immunodetections. In the culture conditions utilized only a proportion of the cells differentiate into glial cells, thus accounting for the presence of transfected cells not expressing GFAP (arrow in panel C).

We also observed that dissociated human CNS precursor cells which were induced to differentiate were also susceptible to transfection (not shown). Under these conditions, efficiency of transfection was only slightly lower (approximately 3%). This result indicates the possibility to analyze gene function in mature human CNS cells.

We were interested in whether an immortalizing oncoprotein could be expressed in human CNS precursor cells. The establishment of immortal human CNS precursor cell lines would indeed represent an invaluable experimental tool in studies of commitment, differentiation and gene function in neural cells of human origin. The availability of oncogenes and the understanding of some aspects of oncogenesis, along with the advent of efficient gene transfer systems, allow for an approach to the establishment of neural cell lines that offers several advantages over the spontaneously transformed and tumor derived cell lines obtained in the past. Oncogenes such as *myc* and Large-T antigen have been shown to exhibit immortalizing abilities without fully transforming the cells. Furthermore, the discovery of mutant alleles of particular oncogenes has allowed the creation of cell lines in which the state of the cells could be regulated by altering the temperature of



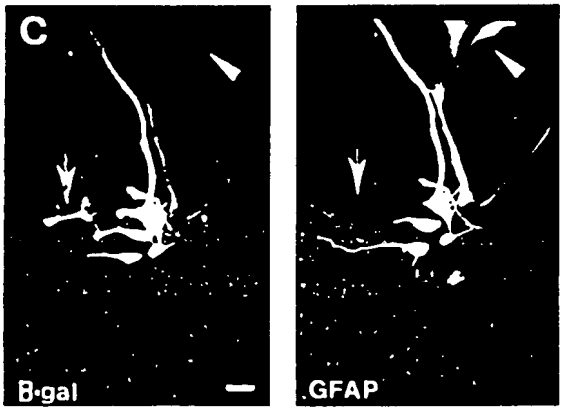


Fig. 2. A: human CNS precursor cells expressing the *LacZ* gene are identified by the presence of a blue precipitate. Five hours after transfection, serum to a final concentration of 2% was added to the cultures for 4 h and thereafter replaced with fresh serum-free medium, the day after fresh growth medium was added. After 24 h cells were fixed with glutaraldehyde 0.1% in PBS + 2 mM $MgCl_2$ for 10 min, rinsed 3 times with PBS and exposed to Triton 0.1%. Transfected cells were identified by exposure to the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; 40 mg/ml) in PBS, 2 mM $MgCl_2$, 0.5 M $K_4Fe(CN)_6$, 0.5 M $K_3Fe(CN)_6$, 2.5% DMSO. (Magnification: 100 ×) Scale bar, 100 μm. B–C: transfected human CNS cells differentiate into neurons (B) and glial cells (C). After transfection the cells were incubated in serum-free medium and allowed to differentiate for 6 days. Fixed cultures were reacted with X-Gal and subsequently exposed to a monoclonal antibody against MAP2 (Boehringer Mannheim). The presence of the antigen was revealed by incubating the cells with a secondary HRP anti-mouse antibody followed by DAB reaction. The arrowheads indicate MAP2 staining in a β-Gal expressing cell (arrow indicates the presence of the blue precipitate). Inset: a transfected bipolar cell with elaborated processes. In C is a double immunohistochemical reaction with monoclonal antibody against β-Gal (left panel) and a polyclonal antibody against GFAP (right panel) followed by FITC-anti-mouse and TRITC-anti-rabbit antibodies (Vector, DBA, Italy). A small cluster of transfected (β-Gal positive) cells that probably had undergone a few rounds of cell divisions is visible. Arrowhead untransfected cells. Right panel GFAP is expressed in a subset of β-Gal positive cells and (arrowhead) in untransfected cells. A GFAP negative transfected cell is also visible (arrow). Scale bar, 10 μm.

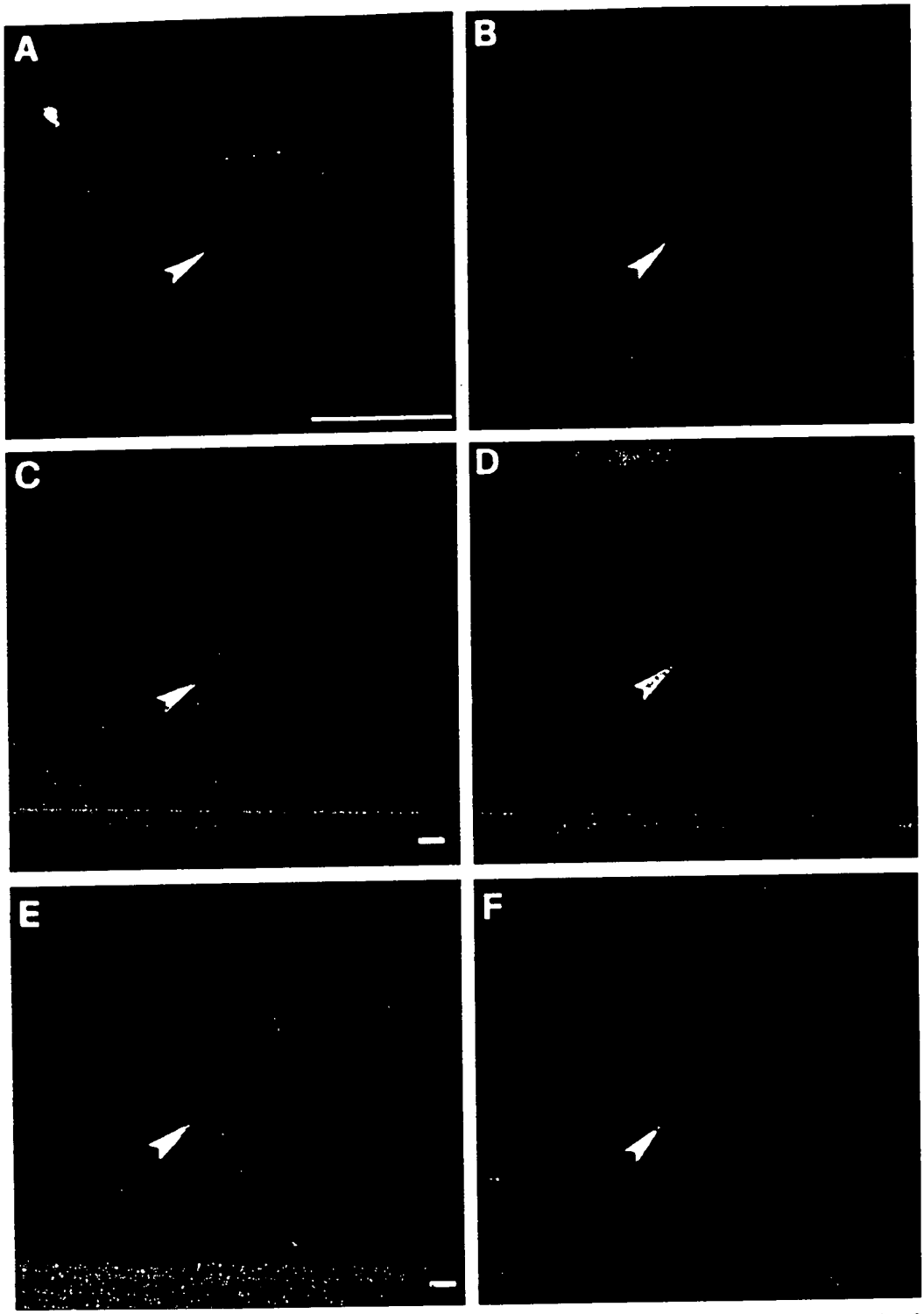


Fig. 3. Expression of Large-T antigen in transiently transfected human fetal CNS stem cells. Cells were fixed with MeOH/acetone for 10 min at 4°C (A and B), 6 (C and D) and 10 (E and F) days after transfection. Cells were permeabilized with Triton 0.4% for 15 min, rinsed with fresh PBS and incubated for 18 h with primary antibody Pab 419 (gift from Ed Harlow). Cells were rinsed several times with PBS and exposed to fluorescein-conjugated secondary antibody for 1 h. After several rinses with PBS, cells were incubated with Hoechst 33258 (5 μg/ml) for nuclear staining. Coverslips were mounted using Permafluor (Halsceintifica, Italy). A, C and E, immunofluorescent staining. The arrows indicate Large-T antigen expressing cells. E, D and F show Hoechst staining. Scale bar, 10 μm.

growth in vitro [12]. Several neuronal and glial cell lines, with immortalizing features, have been developed using this technique. Many of these cell lines have properties of the respective primary cell types from which they derive [1,5,19,21,22]. However, all of these immortalized cell lines have been obtained from rodent CNS, with the exception of a few cell lines generated by immortalizing human astrocytes [17,28].

By following the same experimental procedures identified for the expression of the *LacZ* gene, we delivered the pZipSVtsA58U19 plasmid, carrying the temperature-sensitive allele of SV40 under the control of a retroviral LTR into human CNS precursor cells. Cells were fixed at different time points after transfection and processed for immunohistochemistry utilizing the monoclonal antibody Pab 419 against the Large-T antigen oncoprotein [11]. As shown in Fig. 3, 4 days after transfection, single labeled cells could be identified (Fig. 3A and Fig. 3B) that expressed the oncoprotein at high levels in the cell nuclei. After 6 days, Large-T antigen expressing cells had undergone duplication (Fig. 3C and Fig. 3D), giving rise to doublets of cells that were still positively stained. Ten days after transfection a proportion (up to 10%) of these doublets of cells were still slowly but progressively expanding and colonies of immuno-labeled cells could be identified (Fig. 3E and Fig. 3F).

To our knowledge, these data provide the first indications on the possibility to target exogenous genes into immature human CNS cells using an efficient gene transfer approach alternative to viral-based methodologies. Furthermore, we found that cells transiently transfected with an immortalizing oncogene show high levels of expression of the oncoprotein which is maintained for several days after transfection. This argues in favor of the possibility of obtaining human CNS precursor cell lines with an increased and unlimited proliferative ability which may provide a plentiful source of neurons and glia suitable for transplantation as well as for in vitro studies on the physiology of human brain cells.

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Multipotent CNS Stem Cells Are Present in the Adult Mammalian Spinal Cord and Ventricular Neuroaxis

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Neural stem cells in the lateral ventricles of the adult mouse CNS participate in repopulation of forebrain structures *in vivo* and are amenable to *in vitro* expansion by epidermal growth factor (EGF). There have been no reports of stem cells in more caudal brain regions or in the spinal cord of adult mammals. In this study we found that although ineffective alone, EGF and basic fibroblast growth factor (bFGF) cooperated to induce the proliferation, self-renewal, and expansion of neural stem cells isolated from the adult mouse thoracic spinal cord. The proliferating stem cells, in both primary culture and secondary expanded clones, formed spheres of undifferentiated cells that were induced to differentiate into neurons, astrocytes, and oligodendrocytes. Neural stem cells, whose proliferation was dependent on EGF+bFGF, were also isolated from the lumbar/sacral segment of the spinal cord as well as the third and fourth ventricles (but not adjacent brain parenchyma). Although all of the stem cells examined were similarly multipotent and expandable, quantitative analyses demonstrated that the lateral ventricles (EGF-dependent) and lumbar/sacral spinal cord (EGF+bFGF-dependent) yielded the greatest number of these cells. Thus, the spinal cord and the entire ventricular neuroaxis of the adult mammalian CNS contain multipotent stem cells, present at variable frequency and with unique *in vitro* activation requirements.

Key words: stem cells; spinal cord; ventricles; renewal; multipotent; epidermal growth factor; basic fibroblast growth factor

After formation of the neural tube, a period of prolonged histogenesis, which continues until shortly after birth, results in the formation of the mature CNS. In few instances (discussed below) does neuronal production continue into the adult. Moreover, the adult mammalian CNS shows virtually no capacity for neuronal replacement after injury or disease. Thus, it has been accepted that the adult CNS does not contain stem cells, those specialized cells that participate in cell replacement in tissues that require constant turnover, such as the skin and hematopoietic system (Hall and Watt, 1989; Potten and Loeffler, 1990).

Recently, a series of studies, fueled by evidence for mitotic activity in the subependyma of the forebrain lateral ventricles (Smart, 1961), have lead to the proposition that neural stem cells are indeed present in that region (for review, see Weiss et al., 1996). Morshead and van der Kooy (1992) showed that the subependyma comprised mixed populations of cells, some of which were mitotically active, and that some of the progeny underwent cell death. Subsequently, it was demonstrated that mitotically active cells within the subependyma, when explanted into culture, could generate neurons and glia (Lois and Alvarez-Buylla, 1993) and that adult neuronal precursors in the subependyma migrated to the olfactory bulb to replace dead or dying granule neurons (Lois and Alvarez-Buylla, 1994). Thus, a process that had first been shown to take place in the neonatal brain (Luskin, 1993) persists into adulthood. Coupling these findings and our earlier observation that epidermal growth factor (EGF)-responsive self-renewing cells isolated from the adult striatum could generate neurons and glia *in vitro* (Reynolds and Weiss, 1992), we asked whether these cells were located in the subependyma and were part of the mitotically active population *in vivo*. The use of high [^{3}H]thymidine concentrations to kill cells that were rapidly turning over provided evidence for a relatively quiescent cell with identity to the *in vitro* EGF-responsive cell, whose presumptive role is to repopulate the subependyma (Morshead et al., 1994). These studies support the presence of multipotent stem cells in the mammalian forebrain that participate in repopulation of the subependyma and olfactory bulb.

Persistent neuronal and glial genesis also occurs within the dentate gyrus of the adult rodent hippocampus (Altman and Das, 1965; Bayer et al., 1982; Cameron et al., 1993), and *in vitro* studies demonstrate that basic fibroblast growth factor (bFGF) can support proliferation of adult hippocampal neuronal and glial progenitors (Palmer et al., 1995). The only other report of persistent turnover throughout the adult CNS is that of astroglia (Altman, 1963; Korr, 1980); however, it is unclear whether this turnover occurs in place or is the result of the migration of precursors from the ventricular zone. In this regard, however, there is little evidence for mitotic activity in other ventricles (Chauhan and Lewis, 1979) when compared with that of the subependyma. As opposed to the quiescent nature of the cerebral ventricles (other than the

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lateral ventricles), some mitotic activity has been reported in the adult spinal cord (Adrian and Walker, 1962; Kraus-Ruppert et al., 1975), including a small number of cells that line or are near the central canal (Adrian and Walker, 1962). Frisen and colleagues (1995) demonstrated increased mitotic activity after spinal cord laminectomy resulting in the generation of glia from nestin-positive cells. The authors suggest that these new glia arise from precursors that may be present close to or within the ependymal lining. Some previous studies (for review, see Bruni et al., 1985) have suggested that the ependyma may still contain cells with neuroepithelial potential. Ray and Gage (1994) have demonstrated that bFGF can stimulate embryonic spinal cord neuroblast proliferation; however, actions on adult cells have not been reported. Thus, in the present study we asked whether the adult spinal cord, when dissociated and plated in culture, could yield proliferating multipotent stem cells.

MATERIALS AND METHODS

Primary culture of adult brain tissue. Adult mice (male and female CD1, Charles River, St. Constant, Quebec, Canada) were killed by cervical dislocation. The brain and/or spinal cord were placed in 95%O_2/5%CO_2 oxygenated artificial cerebrospinal fluid [(aCSF) containing 124 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, D-glucose, and penicillin–streptomycin solution 1:25 (Life Technologies, Gaithersburg, MD), pH 7.35, ~280 mOsm] for further dissection. The precise regions and their dissection are illustrated and described in Figure 7 and its legend, respectively. The tissue, regardless of origin, was cut into smaller pieces (~1 mm^3) and transferred into spinner flasks (Bellco Glass) with a magnetic stirrer filled with low Ca^{2+}, high Mg^{2+} aCSF (containing 124 mM NaCl, 5 mM KCl, 3.2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM D-glucose, and penicillin–streptomycin 1:25, pH 7.35, ~280 mOsm), and an enzyme mixture (1.33 mg/ml of trypsin, 0.67 mg/ml of hyaluronidase, and 0.2 mg/ml of kynurenic acid). The stirring tissue suspension was aerated with 95%O_2/5%CO_2 at 32–35°C for 90 min. After this enzymatic incubation period, the tissue was transferred to DMEM/F-12 (1:1; Life Technologies) medium containing 0.7 mg/ml of ovamucoid (Sigma, St. Louis, MO) and triturated mechanically with a fired-narrowed Pasteur pipette. The dissociated cell suspension was centrifuged at 400 rpm for 5 min, and the pellet was washed once and then plated (5000–10.000 viable cells/ml) in noncoated 6-well (2 ml volume) Nunc tissue-culture dishes in media composed of DMEM/F-12 (1:1), including HEPES buffer (5 mM), glucose (0.6%), sodium bicarbonate (3 mM), and glutamine (2 mM). A defined hormone and salt mixture composed of insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM), and sodium selenite (30 nM) was used in place of serum. To the above medium, EGF or bFGF (human recombinant; Chiron Corporation, Emeryville, CA) or both were added at 20 ng/ml. Primary stem cell proliferation was detected after 7–8 d *in vitro* and characterized by the formation of spheres of undifferentiated cells (Reynolds and Weiss, 1992).

Dissociation and perpetuation of EGF+bFGF-generated spheres for clonal analyses. (Schematic representation is given in Fig. 5). To test whether the EGF+bFGF-responsive cell exhibits self-maintenance, two different experiments were carried out: (pathway *2* of Fig. 5) plating of single cells derived from primary EGF+bFGF-generated spheres into 96-well plates and (pathway *3* of Fig. 5) dissociation of single EGF+bFGF-generated spheres. For plating single cells, a single primary EGF+bFGF-generated sphere was collected after 8 d *in vitro*, mechanically dissociated, and serially diluted to yield approximately one to two cells per 10 μl aliquot. A 10 μl aliquot was added to each well of a 96-well plate containing 200 μl of EGF+bFGF-containing medium. Plates were scored 24 hr later. All wells that contained one viable cell were marked, and these wells were rescored 8 d later for the presence of spheres. Single spheres were dissociated by taking a 10–100 μl aliquot of 8 d *in vitro* EGF+bFGF-generated spheres and transferring the spheres into Nucleon 35 mm tissue-culture dishes with EGF+bFGF-containing medium. Under sterile conditions, single spheres were transferred to 500 μl Eppendorf tubes containing 200 μl of medium, triturated 20–40 times, and plated into a 96-well plate. The plates were scored 8 d later for the number of spheres derived from a single sphere.

Differentiation of EGF+bFGF-generated spheres. Eight to ten days after the primary culture or secondary culture (21 d for single-cell-derived spheres), spheres were removed with a pipette, spun down at 400 rpm, and resuspended in EGF+bFGF-containing medium. The spheres were differentiated in single-sphere cultures (pathway *1* of Fig. 5). Single isolated spheres were plated on poly-L-ornithine-coated (15 μg/ml) glass coverslips in individual wells of 24-well Nunclon (1.0 ml/well) culture dishes in DMEM/F-12 medium with the hormone and salt mixture and EGF+bFGF. Medium was not changed for the rest of the experiment. Coverslips were processed 21–25 d later for indirect immunocytochemistry.

Antibodies. Rabbit antiserum to nestin (Rat 401; 1:1500) was a gift from Drs. M. Marvin and R. McKay; a mouse monoclonal antibody against the 168 kDa neurofilament protein (clone RMO 270; 1:50) was generously supplied by Dr. V. Lee; rabbit antiserum to glial fibrillary acidic protein (GFAP; 1:1000) was a gift from Dr. L. Eng; mouse monoclonal antibody (IgM) to O4 (1:20) was a gift from Dr. M. Schachner; mouse monoclonal antibody to MAP-2 was from Boehringer Mannheim (Indianapolis, IN); and mouse monoclonal antibody to β-tubulin (Type III; 1:1000) was from Sigma. Rabbit polyclonal antisera to Substance P (1:1000) was from Incstar, and to GABA (1:3000) was from Chemicon. Fluorescein-conjugated and rhodamine-conjugated affinipure goat antibody to mouse IgG, rhodamine-conjugated affinipure goat antibody to rabbit IgG, and AMCA-conjugated affinipure goat antibody to mouse IgM were obtained from Jackson ImmunoResearch (West Grove, PA).

Immunocytochemistry. Indirect immunocytochemistry was carried out with spheres attached to glass coverslips, either immediately after plating (for nestin) or after 21–25 d *in vitro* (for triple-labeling and for neuronal phenotypes). Coverslips were fixed in 4% paraformaldehyde (in PBS, pH 7.2) for 30 min, followed by three (10 min each) washes in PBS, pH 7.2. Nestin, Substance P, or GABA antisera were diluted in PBS/10% normal goat serum/0.3% Triton X-100 and incubated with the coverslips for 2 hr at 37°C. Coverslips were washed three times (10 min each) in PBS and incubated in appropriate secondary antibodies (1:100) for 30 min at 37°C. For the triple-labeling experiments, cells were permeabilized briefly for 5 min (0.3% Triton X-100/PBS) after fixation, followed by the addition of the neuron-specific monoclonal antibody to either MAP-2 or NFM (IgG) together with polyclonal antiserum to GFAP. Appropriate secondary antibodies were added, followed by incubation with monoclonal antibody to O4 (IgM), and a goat anti-mouse IgM specific secondary (AMCA) was used to visualize the O4 antibody. It is noteworthy that the permeabilization procedure renders the normal uniform staining of the extracellular antigen O4 (Reynolds and Weiss, 1993) to a punctate representation (Reynolds and Weiss, 1996; this study). Coverslips received three (10 min each) washes in PBS and were rinsed with water, placed on glass slides, and coverslipped with Fluorsave as the mounting medium. Fluorescence was detected and photographed on a Zeiss photomicroscope with Kodak T-Max 400 film.

RESULTS

Multipotent cells that respond to EGF+bFGF can be isolated from the adult thoracic spinal cord

In previous studies (Reynolds and Weiss, 1992; Morshead et al., 1994), we found that EGF induced the proliferation of multipotent, self-renewing, and expandable stem cells that were isolated from the adult subependymal cell layer of the forebrain. On proliferation, these cells formed spheres of undifferentiated cells that could generate neurons and glia. Thus, we asked whether similar cells could be isolated from the adult spinal cord. Adult thoracic spinal cord was dissected, enzymatically dissociated, and plated in the presence of EGF (20 ng/ml) or bFGF (20 ng/ml). After 8 d *in vitro*, cells cultured in the presence of EGF showed no evidence of the characteristic spheres of proliferating cells. In the presence of bFGF, very small clusters of cells were found; however, these clusters could not renew (only 15% produced one secondary sphere) or expand (none produced more than one secondary sphere). When EGF and bFGF were combined, however, large self-renewing and expandable spheres were generated (Fig. 1). Quantitative analysis showed that 8.6 ± 3.4 spheres/5000 viable cells were generated. These spheres were similar to those isolated from the subependymal cell layer of the forebrain in that

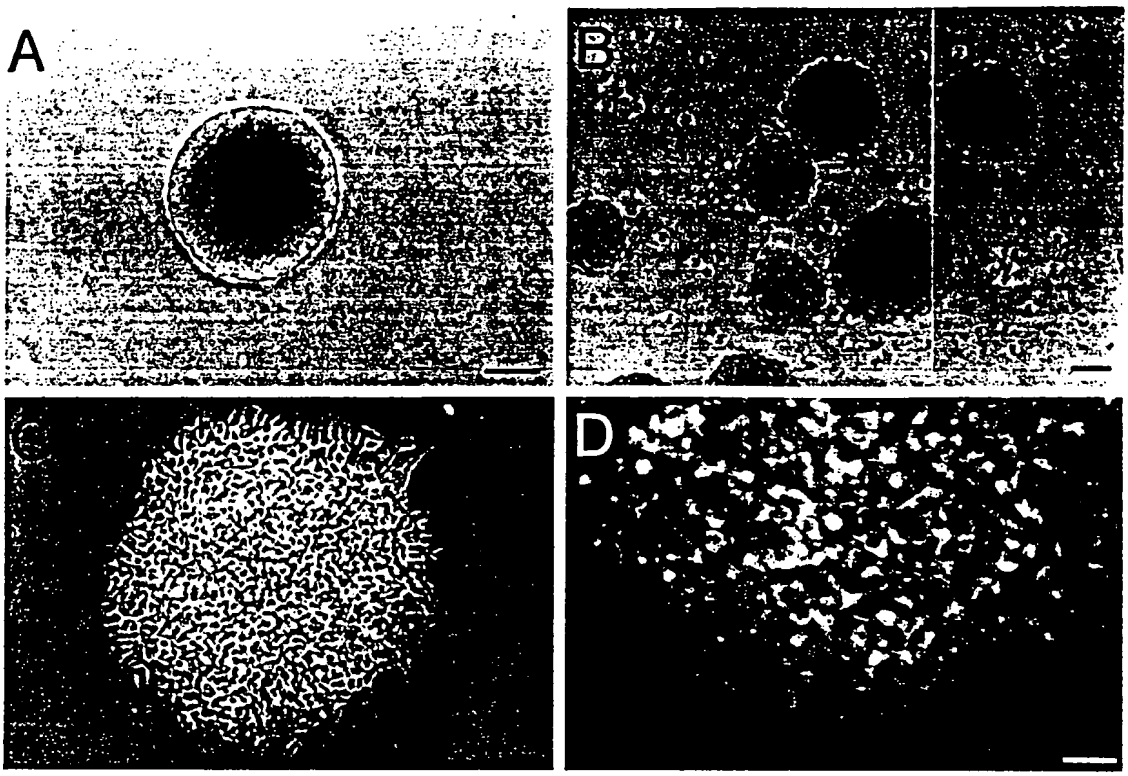

Figure 1. Characteristics of spheres generated from isolated cells of the adult thoracic spinal cord. *A*, *B*, An example of a single sphere generated from the adult thoracic cord (*A*), which was dissociated into single cells that yielded close to 300 spheres 1 week later, some of which are illustrated in *B*. *C*, *D*, Spheres generated from the adult thoracic spinal cord contained no differentiated cells; however, virtually all cells within these spheres (*C*) expressed nestin (*D*), an intermediate filament characteristic of undifferentiated neuroepithelial cells. Scale bars: *A*, 100 μm; *B*, 50 μm; *C*, 50 μm; *D*, 30 μm.

a single sphere (Fig. 1*A*) could be dissociated and replated under identical conditions to yield more than one of itself (Fig. 1*B*). Quantitative analysis showed that on average a single primary sphere yielded 127 ± 14 secondary spheres. Also similar to that observed in forebrain-derived cultures, both primary and secondary spheres contained undifferentiated cells, as determined by the expression of nestin immunoreactivity (Fig. 1*C*,*D*) and the absence of antigens characteristic of differentiated neural cells (data not shown).

We next asked whether the EGF+bFGF-generated spheres could yield differentiated neural cells when plated on a poly-L-ornithine substrate. Single spheres were transferred onto polycation-coated glass coverslips in the continued presence of EGF+bFGF and cultured for an additional 21 d *in vitro*. The single-sphere cultures were then fixed and processed for triple-label immunocytochemistry. Antibodies to MAP-2 or NFM were used to identify neurons, whereas antiserum to GFAP and antibody to O4 were used to identify astrocytes and oligodendrocytes, respectively. When MAP-2 was the antigen examined for neuronal identity, every sphere was found to contain the three principal neural cell types (106/106 spheres from 23 separate primary cultures). An example is illustrated in Figure 2. As outlined in Materials and Methods, permeabilization renders the normally uniform staining with O4 to a punctate representation. This is illustrated in Figure 3, whereby an example of a nonpermeabilized O4-immunoreactive cell (Fig. 3*A*; typical oligodendrocyte morphology) is contrasted with a permeabilized O4-immunoreactive cell (Fig. 3*B*,*C*; selective, punctate staining) from a sister culture. Without such permeabilization, we could not clearly detect or distinguish neurons from the other two cell types when the three were examined simultaneously. Under these experimental conditions, 1.0 ± 0.2% of total cells were identified as neurons, 0.3 ± 0.1% as astrocytes, and 0.7 ± 0.2% as oligodendrocytes (n = 20). The majority of the remaining cells was immunoreactive for nestin (data not shown).

When NFM was the neuronal antigen, 13 of 15 spheres (from three separate primary cultures) examined showed the three cell types. An example of NFM immunoreactivity illustrated in Figure 4*B* shows the thin fibers that displayed immunoreactivity; cell bodies were rarely labeled. We next examined the presence of distinct neuronal phenotypes in single-sphere cultures derived from the thoracic spinal cord. Every sphere examined (37/37 spheres from six separate primary cultures) contained GABAergic neurons (example given in Fig. 4*E*,*F*). Neurons that were immunoreactive for substance P were also found (data not shown). Other neurotransmitter phenotypes, e.g., serotonin, tyrosine hydroxylase, and choline acetyltransferase, were not detected in these single-sphere cultures. Thus, the neurotransmitter phenotype of thoracic spheres was similar to that of both embryonic (Reynolds et al., 1992; Ahmed et al., 1995) and adult (Reynolds and Weiss, 1992) EGF-generated forebrain spheres.

Clonal analysis demonstrates that the thoracic spinal cord cells that proliferate in response to EGF+bFGF are neural stem cells

Recently, we and others have developed criteria for demonstrating that a proliferating adult CNS cell is a stem cell (Gritti et al., 1996; Reynolds and Weiss, 1996). This involves examining the expansion of secondary clones derived from primary proliferating cells. These criteria are illustrated schematically in Figure 5. In

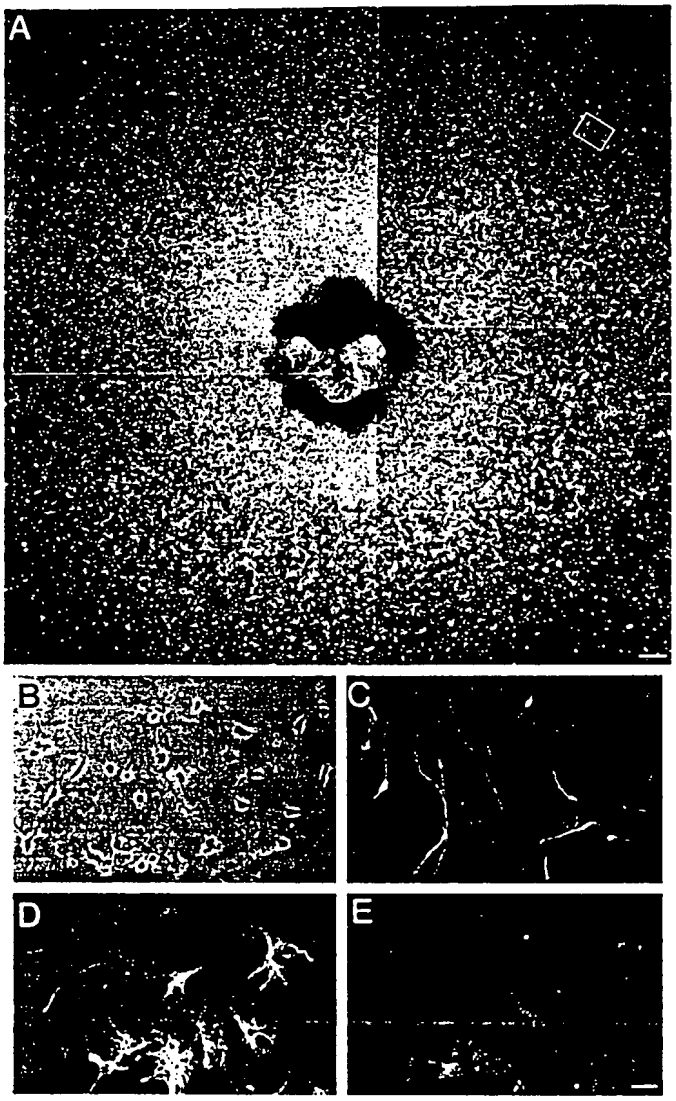

Figure 2. Single spheres derived from the adult thoracic spinal cord yielded neurons, astrocytes, and oligodendrocytes. *A*, A single, isolated sphere was transferred onto poly-L-ornithine-coated glass coverslips, cultured for 3 weeks in the presence of EGF+bFGF, fixed, and processed for indirect immunocytochemistry. *B–E*, Triple-label immunocytochemistry of the sphere in *A*, illustrating the cells within the box (*B*), shows (*C*) MAP-2-, (*D*) GFAP-, and (*E*) O4-immunoreactive cells, with the morphology of neurons, astrocytes, and oligodendrocytes, respectively. Scale bars: *A*, 200 μm; *B–E* (shown in *E*), 20 μm.

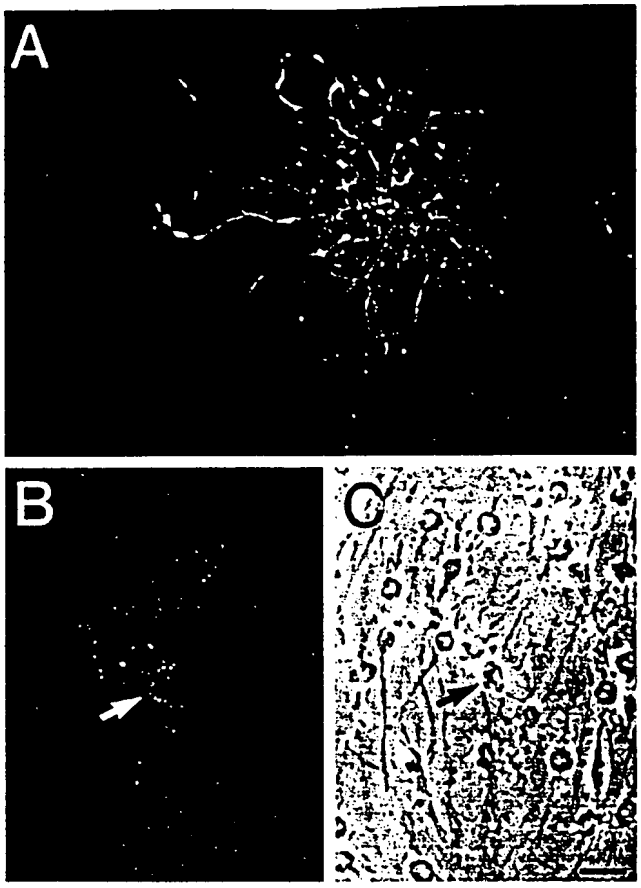

Figure 3. Examples of O4-immunoreactive cells in plated adult thoracic spinal cord spheres, with and without permeabilization. *A*, An O4-immunoreactive cell in a fixed, but nonpermeabilized, single thoracic sphere culture, demonstrating typical oligodendrocyte morphology. *B, C*, In a fixed and permeabilized sister culture, a single oligodendrocyte (*arrow*) is specifically stained in a punctate fashion with the O4 antibody. Scale bar, 20 μm.

this scheme, a neural stem cell can self-renew and expand, and the progeny of the secondary expanded clones should exhibit the same phenotype as the primary cells. Thus, we tested the spheres derived from thoracic spinal cord, in the presence of EGF+bFGF, to determine whether they would satisfy these criteria. First, we showed that single cells, derived from single primary spheres, could proliferate and form spheres that generate neurons, astrocytes, and oligodendrocytes (Fig. 6). Next, we compared the phenotype potential of primary spheres and secondary spheres derived from single-cell culture or total single-sphere dissociations. The results are summarized in Table 1. When primary spheres were examined for their ability to generated neurons, astrocytes, and oligodendrocytes, all were found to be tripotential. This was also true for secondary spheres, both those generated through single-cell culture (11/11 spheres, six primary cultures) and those generated through single-sphere dissociation (79/79 spheres, 15 primary cultures). The ability to self-renew, expand, and maintain the potential to produce the three major cell types supports the contention that the cells from the thoracic spinal cord that proliferate in response to EGF+bFGF are stem cells.

Neural stem cells are present in other regions of the spinal cord and in the third and fourth ventricles

The presence of self-renewing stem cells in the thoracic spinal cord, whose proliferation depended on the combined actions of EGF and bFGF, prompted us to examine whether similar cells reside in other spinal cord regions as well as line other ventricles. We hypothesized that such cells would line ventricles, given previous findings that in the forebrain neural stem cells could be isolated only from tissue that contained the subependymal cell layer (Morshead et al., 1994). Thus, we compared the frequency and growth factor dependence of putative neural stem cells, isolated from the lateral, third, and fourth ventricles (with adjacent parenchyma as a suspected negative control) and from the thoracic and lumbar/sacral segments of the spinal cord. Separating central canal from adjacent spinal cord tissue was technically not possible. The precise dissection is outlined in Figure 7. We plated equivalent numbers of cells from the various regions and

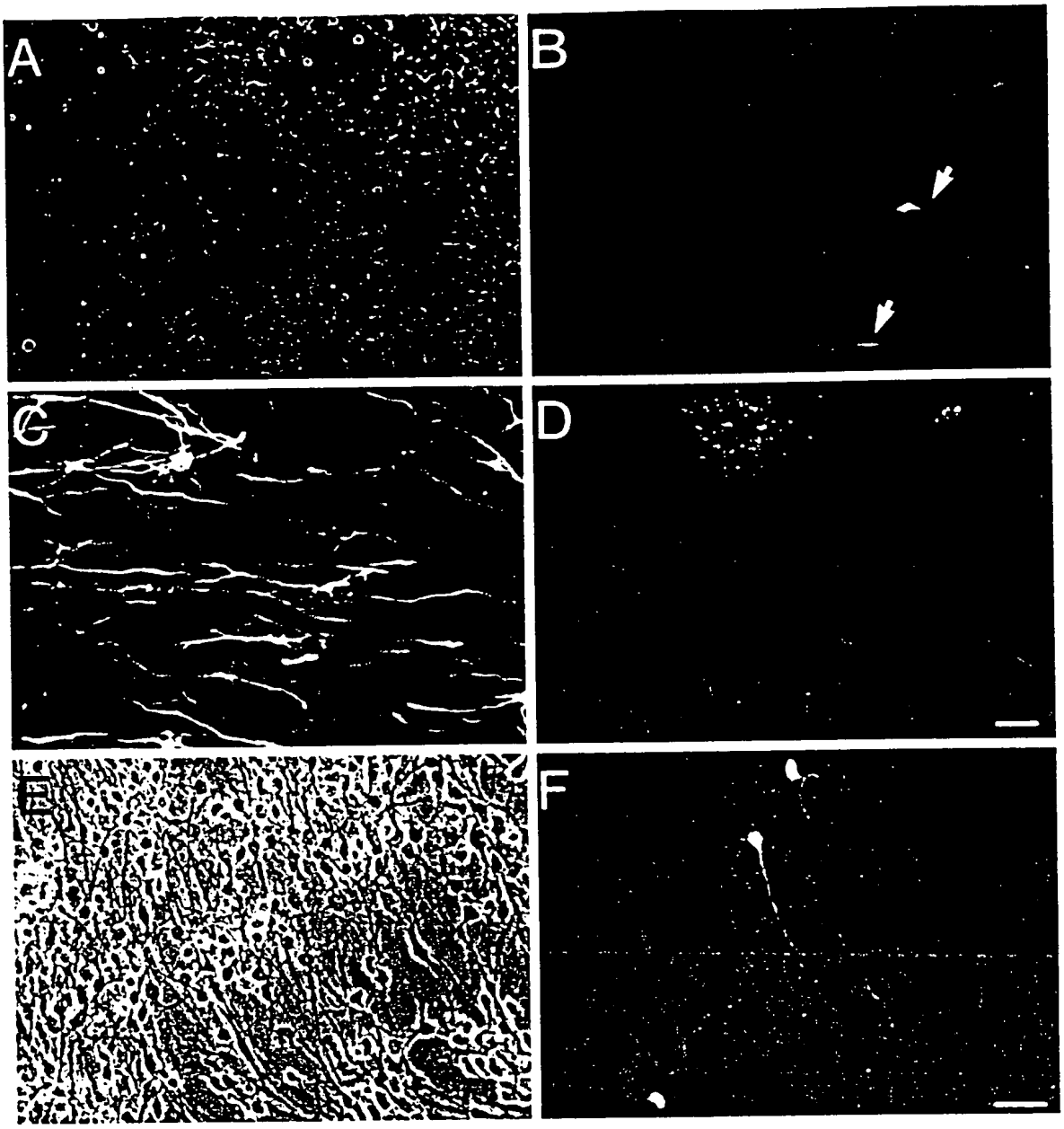

Figure 4. Further characterization of the phenotypes of cells derived from stem cells of the thoracic spinal cord. *A–D*, Triple-label immunocytochemistry of single spheres derived from the thoracic spinal cord, after 3 weeks of plating on poly-L-ornithine. *A*, Representative field shows (*B*) neurofilament M (160 kDa; *arrows*), (*C*) GFAP, and (*D*) O4 immunoreactivity, characteristic of neurons, astrocytes, and oligodendrocytes, respectively. *E*, *F*, The principal neuronal phenotype detected, after 3 weeks of plating on poly-L-ornithine, was GABA. Indirect immunocytochemistry of a representative field (*E*) shows cells with neuronal morphology that were GABA-immunoreactive (*F*). Scale bars: *A–D* (shown in *D*), 20 μm; *E*, *F* (shown in *F*), 30 μm.

counted the numbers of spheres generated per 5000 viable cells plated, in the presence of EGF or bFGF alone or together. Only those spheres that could be subcloned (as described above and illustrated later in Table 3) were counted. Our results, shown in Table 2, suggest that stem cells with different growth requirements and frequencies are present throughout the entire ventricular neuroaxis. First, only cells within the lateral ventricles yielded self-renewing and expandable spheres in response to EGF (26.7 ± 3.7 spheres/5000 cells). An identical number (26.8 ± 4.5) was found when EGF and bFGF were combined; however, in both of the other ventricular regions tested, neither EGF nor bFGF alone was sufficient to induce the formation of self-renewing, expandable spheres. Moreover, there was a decreasing frequency from the lateral to the third and then fourth ventricle. The cultures of the third ventricle differed from all other regions in one regard: spheres took twice as long to form (14–16 d vs 7–8 d for all other regions). In all cases, no spheres were generated when the adjacent parenchyma (as illustrated in Fig. 7) was cultured under identical conditions.

When comparing the thoracic and lumbar/sacral regions of the spinal cord, where again self-renewing, expandable spheres were generated only with EGF+bFGF, we found that the greatest number of spheres was generated from cells of the lumbar/sacral cord (32.6 ± 3.2). This represented an approximately fourfold greater frequency when compared with the thoracic cord. Moreover, this represented the greatest frequency in relation to all

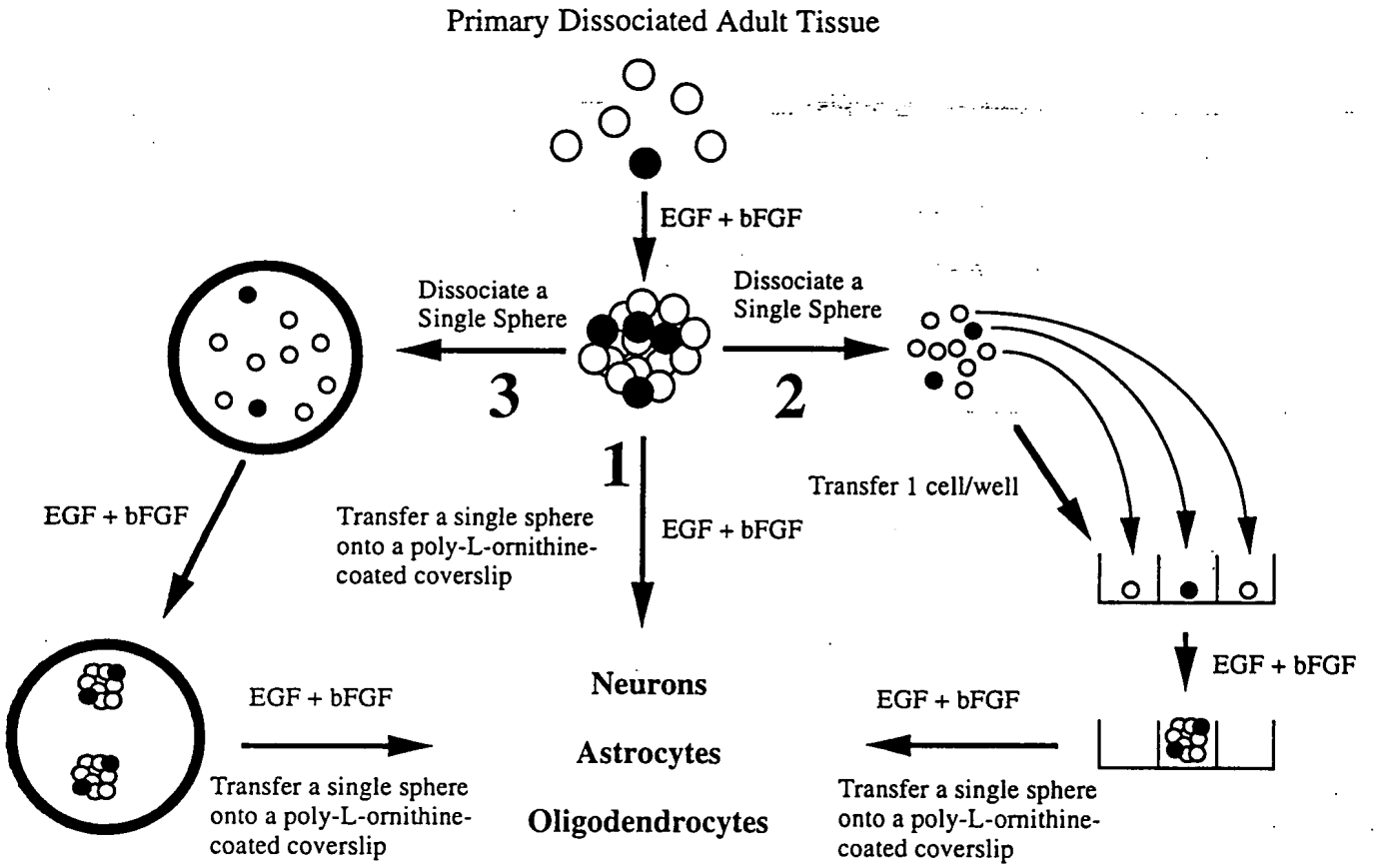

Figure 5. Schematic representation of approaches used to establish adult spinal cord stem cell proliferation, self-renewal and expansion, and production of neurons, astrocytes, and oligodendrocytes. The experimental approaches to demonstrating self-renewal and expansion of stem cells in response to EGF+bFGF are shown. When primary, dissociated adult cells are exposed to EGF+bFGF, spheres of undifferentiated cells are generated. (1) Differentiation of single primary spheres results in the production of neurons, astrocytes, and oligodendrocytes. (2) Dissociation of single primary spheres into single cells, which are plated after serial dilution as *1 cell/well*, generates clonally derived secondary spheres. Differentiation of single secondary spheres results in the production of neurons, astrocytes, and oligodendrocytes. (3) Dissociation of single primary spheres into single cells, all of which are plated into one well, results in more than one secondary sphere. Once again, differentiation of these single secondary spheres results in the production of neurons, astrocytes, and oligodendrocytes.

regions tested. Given the differing tissue dissections, however, comparisons with the ventricles would be difficult. Furthermore, it may be argued that with differing thicknesses/enlargements of the spinal cord, even comparisons between the segments may be misleading. Thus, we compared the frequency of EGF+bFGF-generated spheres from cervical, thoracic, or lumbar/sacral spinal cord, this time normalizing for length of spinal cord. Our findings, shown in Figure 8, confirm the results given in Table 2. First, the comparison between thoracic and lumbar/sacral cord showed a three- to fourfold greater frequency of spheres in the latter regions. Furthermore, the frequency of spheres in the cervical cord (not previously examined) was the lowest of all of the spinal cord regions.

Although in all previous studies the formation of spheres was generally indicative of it being derived from stem cells, e.g., tripotential cells with self-renewal and expandable properties, we wished to confirm this to be the case for all of the regions examined. Thus, we compared the ability of spheres generated by EGF+bFGF, from the five regions examined, to self-renew/expand and generate the three cell types. The results are shown in Table 3. Primary (n = 9–23 individual cultures) and secondary (n = 4–15 individual cultures) spheres were examined in the manner illustrated in pathways 1 and 3 of Figure 5. All primary spheres, regardless of region of origin, displayed an impressive ability to expand, yielding ~79-127 secondary spheres. The differences between regions were not statistically significant. Furthermore, in virtually all cases (430/433 individual spheres), neurons, astrocytes, and oligodendrocytes were generated in primary and secondary spheres. The three secondary spheres of the third ventricle contained neurons and astrocytes only. Therefore, using the criteria established above for embryonic cells that generated spheres (Reynolds and Weiss, 1996) and for thoracic spheres (Figs. 1–5, Table 1), it is reasonable to conclude that the spheres generated from all of the ventricles and spinal cord regions in response to EGF+bFGF are derived from multipotential, self-renewing stem cells.

DISCUSSION

The results of this study suggest that multipotent stem cells are present in the adult spinal cord and throughout the entire ventricular neuroaxis. Although stem cells isolated from the forebrain subependymal zone proliferate and expand in response to EGF alone (Reynolds and Weiss, 1992; Morshead et al., 1994), the stem cells of the third and fourth ventricles and spinal cord require the combined actions of EGF and bFGF. All of these stem

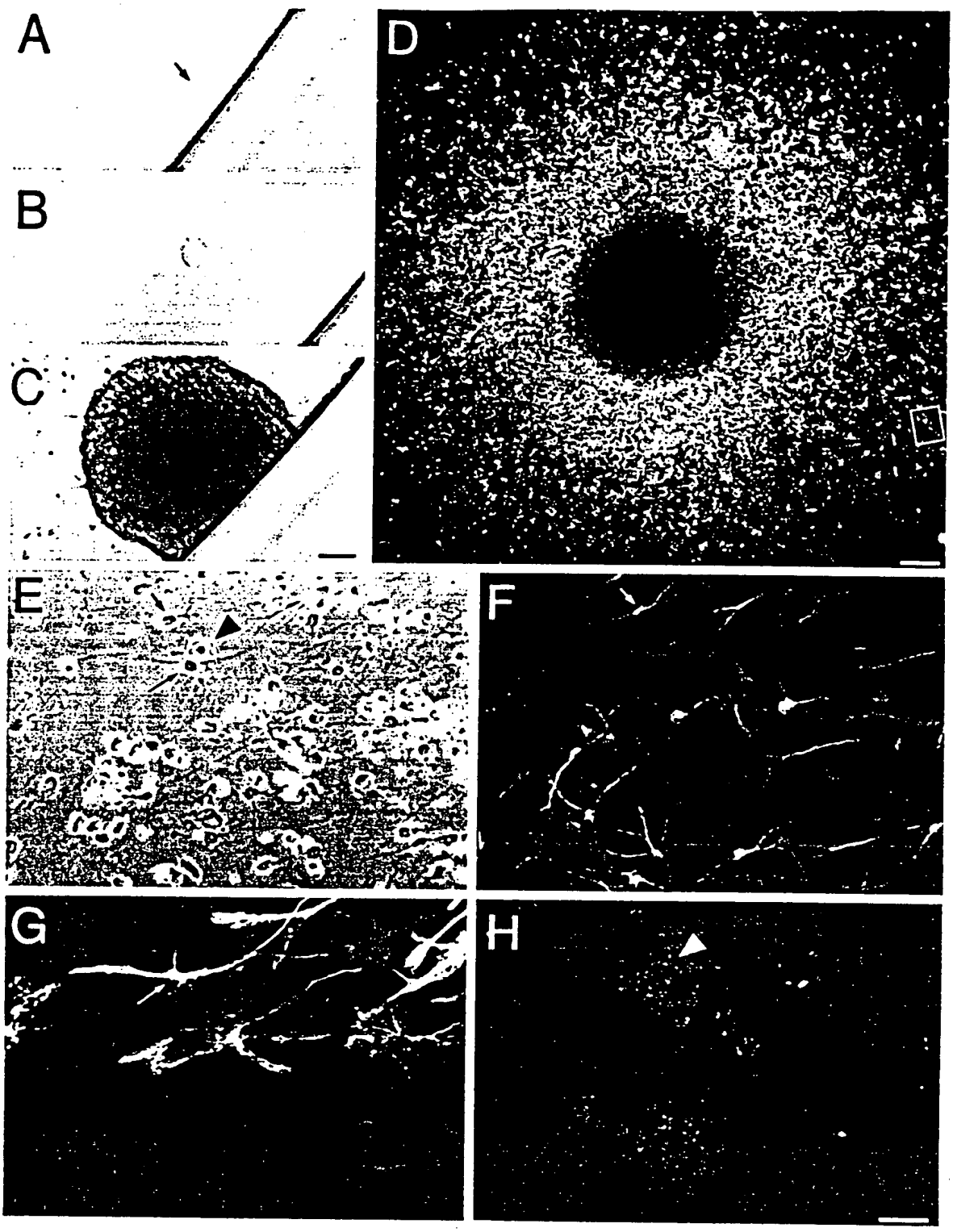

Figure 6. Primary spheres from the adult thoracic spinal cord give rise to clonally derived, multipotent secondary spheres. *A–H*, Multipotent secondary spheres are derived from a single cell. A single cell (*arrow*) dissociated from a primary sphere (*A*) after 24 hr. After 5 d *in vitro* (*B*), the cell has begun to proliferate and has formed a large sphere after 14 d *in vitro* (*C*). The sphere was transferred to a glass coverslip and cultured in the presence of EGF+bFGF. After 3 weeks (*D*), the sphere was processed for indirect immunocytochemistry. The *box* designates the field (*E*) that, through triple-labeling for MAP-2, GFAP, and O4 immunoreactivities, revealed the presence of neurons (*F*, *short arrow*), astrocytes (*G*, *long arrow*), and oligodendrocytes (*H*, *arrowhead*), respectively. Scale bars: *A–C* (shown in *C*), 50 μm; *D*, 140 μm; *E–H* (shown in *H*), 30 μm.

cells share two hallmark properties: self-renewal/expansion and multipotency, as defined by the production of neurons, astrocytes, and oligodendrocytes by single stem cells (Gritti et al., 1996; Reynolds and Weiss, 1996; Weiss et al., 1996). Taken together with previous studies of adult neural stem cells, however, these findings suggest that heterogeneity likely exists between (1) pri-

Table 1. Multipotency of primary and renewed adult thoracic spinal cord stem cell-derived spheres

Experimental protocol	Number of independent primary cultures	Frequency of spheres containing neurons, astrocytes, and oligodendrocytes
Primary culture	23	106/106
Single cell culture	6	11/11
Single sphere dissociation	15	79/79

The experimental protocols correspond to those outlined schematically in Figure 5. Indirect immunocytochemistry for the three neural antigens is described in Materials and Methods.

mary stem cells (those removed from the brain) and the secondary stem cells they produce in culture and (2) stem cells in different ventricular regions, which may be related to their origin and/or functional roles *in vivo*.

A noteworthy finding in this study was the combined actions of EGF and bFGF in inducing proliferation of stem cells from the spinal cord and third and fourth ventricles. Basic FGF has been reported to cooperate with other signals in allowing the long-term renewal of both pluripotential embryonic stem cells (Matsui et al., 1992) and O-2A glial progenitor cells (Bogler et al., 1990), and thus the cooperative nature of the response is not unusual. Our result, however, is in contrast to previous reports of EGF (Reynolds and Weiss, 1992) and bFGF (Gritti et al., 1996) individually as mitogens for subependymal/forebrain stem cells. Two questions arise immediately. First, why was bFGF reported to be ineffective in the first report (Reynolds and Weiss, 1992) of adult forebrain stem cells? Second, what can one conclude/propose regarding the *in vitro* actions of bFGF and/or EGF on adult spinal cord stem cells? The first question can be answered by comparing findings reported in this study for thoracic spinal cord stem cells with those of the lateral ventricle/forebrain (Reynolds and Weiss, 1992; Gritti et al., 1996). In the cultures of thoracic spinal cord, although EGF alone does not produce any spheres, bFGF alone will produce very small spheres. These bFGF spheres can be dissociated; however, they will never produce more than one secondary sphere, and only 15% of the time do they actually

Table 2. Frequency and growth factor dependence of primary multipotent stem cell-derived spheres

Brain region	Numbers of self-renewing, expandable spheres/5000 cells plated (mean ± SEM) EGF	bFGF	EGF + bFGF
Lateral ventricle	26.7 ± 3.7	–	26.8 ± 4.5
Third ventricle	–	–	6.1 ± 1.4[a]
Fourth ventricle	–	–	1.0 ± 0.3
Thoracic cord	–	–	8.6 ± 3.4
Lumbar/sacral cord	–	–	32.6 ± 3.2

The brain regions indicated are those shown schematically in Figure 7. The data are the mean ± SEM of spheres formed after 8 d in culture in at least four independent culture preparations, each condition performed in duplicate. Basic FGF was tested in the absence of added heparin [see Discussion and Gritti et al. (1996)].

[a] Counted after 14–16 d.

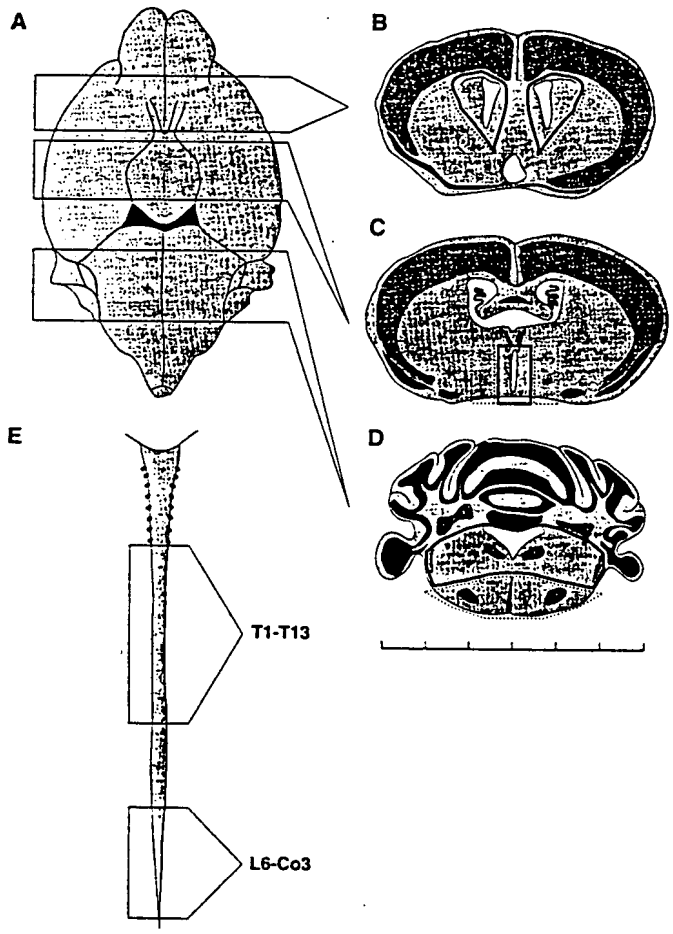

Figure 7. Regions of the adult CNS examined for the presence of growth factor-responsive stem cells. *A–D*, Ventral view of the adult mouse brain (*A*), illustrating the coronal sectioned regions that were used to dissect lateral ventricle (*B*), third ventricle (*C*), and fourth ventricle (*D*). *Dark lines* illustrate the regions considered ventricular, whereas *stippled lines* illustrate nonventricular regions of the same thick section. *E*, Adult mouse spinal cord, illustrating the regions dissected as thoracic (*T1–T13*) and lumbar/sacral (*L6–Co3*). As detailed in Results, stem cells were isolated from all ventricular regions examined but not from the adjacent parenchyma. Scale bar: each graduation is 1 mm.

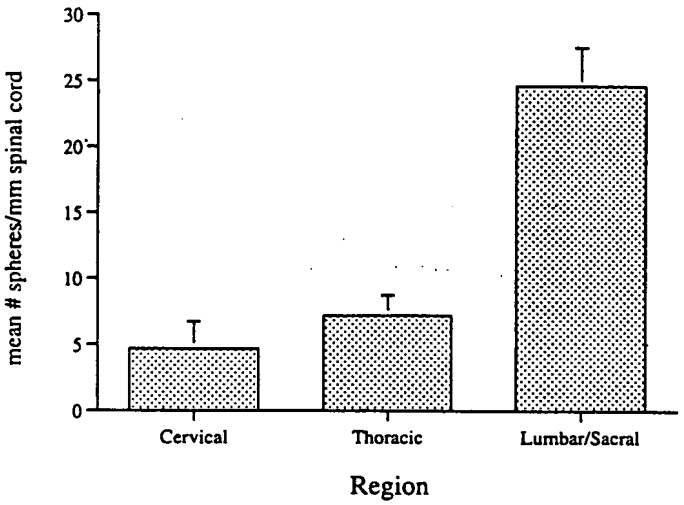

Figure 8. The *in vitro* generation of spheres derived from isolated cells of the adult spinal cord is greatest in the *Lumbar/Sacral* segment. The number of spheres generated in the presence of EGF+bFGF *in vitro* was determined for the three regions of the spinal cord indicated and was normalized to the length of spinal cord tissue dissected. The data represent the mean ± SEM of duplicate determinations in six independent culture preparations.

Table 3. Expansion and multipotency of growth factor-generated spheres derived from various brain regions

Brain region	No. 2° spheres (mean ± SEM)	No. of cultures	No. of spheres	No. N+A+O
Lateral ventricle	79 ± 20	1° 9	37	37
		2° 4	9	9
Third ventricle	84 ± 21	1° 10	33	33
		2° 6	33	30[a]
Fourth ventricle	107 ± 22	1° 12	37	37
		2° 9	54	54
Thoracic cord	127 ± 14	1° 23	106	106
		2° 15	79	79
Lumbar/sacral cord	110 ± 11	1° 9	34	34
		2° 5	11	11

The left-hand column is a comparison of the expandability of primary spheres, generated in the presence of EGF + bFGF, from the indicated regions. No. 2° spheres refers to the numbers of secondary spheres counted in a well where all of the dissociated cells of a primary sphere had been seeded. The data for those single-sphere dissociations are the mean ± SEM of 10–20 primary spheres dissociated within each region. The remaining three columns refer to the multipotential nature of primary spheres and their progeny, the secondary spheres produced through subcloning. The total numbers of individual experiments and spheres examined are given. 1°, Primary culture; 2°, secondary culture.

[a] Three spheres contained only neurons and astrocytes. N+A+O, Neurons + astrocytes + oligodendrocytes.

renew themselves. We found that forebrain stem cells responded in a virtually identical fashion to bFGF alone (J. Hewson and S. Weiss, unpublished observations). Gritti et al. (1996) added heparin to their cultures of forebrain stem cells, and the actions of bFGF changed dramatically, resulting in the production of larger spheres with the potential to expand. The lack of this cofactor serves to explain why we reported that bFGF did not mimic EGF in inducing proliferation and self-renewal/expansion of stem cells in our early forebrain study (Reynolds and Weiss, 1992).

The second and perhaps more intriguing question, regarding the respective roles/actions of EGF versus bFGF in neural stem cell proliferation, points to differences between primary stem cells and their progeny, the secondary stem cells produced in culture. After this study reached completion, we read the study of Gritti et al. (1996), which reported successful subcloning of bFGF+heparin-responsive stem cells in cultures of adult forebrain, similar to what we reported earlier with EGF-stimulated adult forebrain stem cells (Reynolds and Weiss, 1992). When we compared the generation of thoracic spinal cord stem cell-generated spheres in the two growth factor combinations, e.g., EGF+bFGF versus bFGF+heparin, we found that both conditions produced similar expandable, multipotential spheres (C. Dunne and S. Weiss, unpublished observations). Considering the observation that EGF alone gives no spheres and bFGF alone gives very small spheres that cannot expand, we propose the following. Our working hypothesis is that primary thoracic spinal cord stem cell division is stimulated by activation of the bFGF receptor. Adequate heparin, likely in its proteoglycan form, is present on the primary cells to support proliferation (Spivak-Kroizman et al., 1994) in the absence of any added in culture. The proliferation of secondary stem cells can be stimulated by either bFGF (requiring heparin) or EGF. In support of this hypothesis are preliminary results whereby primary 8-d-old spheres generated in either EGF+bFGF or bFGF+heparin were successfully subcloned in EGF alone (C. Dunne and S. Weiss, unpublished observations).

Given the model proposed above for thoracic spinal cord stem cells and noting the *in vitro* response of forebrain stem cells to EGF alone (Reynolds and Weiss, 1992; this study), it seems plausible to conclude that the primary stem cells isolated from the lateral ventricles and spinal cord differ in their response to growth factors. Might this be related to different mitotic activities within these distinct adult CNS regions? As outlined in the introduction, the principal region of mitotic activity in the adult brain is the subepedymal cell layer of the lateral ventricles (Smart, 1961; Morshead and van der Kooy, 1992). Other than the subependyma, only the central canal (ependyma) of the spinal cord has been demonstrated to exhibit significant mitotic activity (relative to any other ventricles) in the adult (Adrian and Walker, 1962; Kraus-Ruppert et al., 1975). These mitotic activities, however, differ remarkably with respect to the location and number of cells, normal fate of the progeny, and response to injury. First, in the lateral ventricles, the vast majority of significant constitutive proliferation is within the subependyma (Smart, 1961; Morshead and van der Kooy, 1992), whereas in the spinal cord it is the ependyma of the central canal and not the subependyma that contains most, albeit few in number, of the mitotically active cells (Adrian and Walker, 1962). Moreover, even within the ependyma, the labeling index for the central canal was 8%, whereas that of the forebrain lateral ventricles was 22% (Kraus-Ruppert et al., 1975). Second, although Lois and Alvarez-Buylla (1994) convincingly demonstrated that mitotically active cells of the subependyma migrate rostrally to the olfactory bulb to produce new neurons, previous studies of the spinal cord found no evidence for new neurons or for migration of the mitotically active cells of the ependyma (Adrian and Walker, 1962). Finally, it is interesting to note how these two mitotically active regions respond to injury. When the striatal parenchyma adjacent to the lateral ventricles is injured by a kainic acid lesion or a knife cut, there is an increase in mitotic activity in the subependyma, but no new cells migrate into the injured areas (Morshead and van der Kooy, 1992) (D. van der Kooy, personal communications). On the other hand, Frisen and colleagues (1995) recently showed that after laminectomy, new astrocytes appear to migrate from a region adjacent to the central canal to contribute to the glial scar. These authors concluded that a progenitor or stem cell population may indeed be present near or in the central canal, which can be identified by nestin expression, and it is this population that is mobilized and recruited to injury sites as part of the formation of the glial scar. In summary, the mitotic activities of the lateral ventricles/subependyma and central canal differ in many respects. Thus, it is plausible that two populations of stem cells, which clearly subserve at least two

distinct functions in the adult *in vivo*, are present in the lateral ventricles and spinal cord, respectively. Consequently, when they are isolated *in vitro*, it is perhaps not unexpected that these two populations respond differently to growth factors.

In addition to those issues discussed above, at least three additional questions remain unanswered, the latter two specifically regarding the stem cells isolated from the adult spinal cord. (1) How do our findings relate to those of Palmer and colleagues (1995), who have generated bFGF-dependent long-term cultures of neuronal and glial progenitors from both ventricular and nonventricular adult brain regions? It is possible that different culture conditions by Palmer et al. (1995), e.g., use of serum and higher concentrations of bFGF, may allow for stimulation of growth from nonventricular regions, something we never observed in our cultures whether EGF+bFGF or bFGF+heparin was used. It is difficult to compare the exact nature of the cells that respond in the bFGF-dependent cultures, because clonal analyses were not performed; however, we concur with the authors' speculation that one difference between the ventricular and nonventricular zone might be the primitive nature of the cells. Thus, the ventricular zones likely contain the most primitive stem-like cells, those isolated in our study. The nonventricular zones (parenchyma) may contain more restricted progenitors, many of which may require more complex signaling to be mobilized. (2) Do the spinal cord stem cells have the potential to produce motor neurons? Although Gritti et al. (1996) report the presence of ChAT-immunoreactive neurons in cultures of forebrain stem cell progeny, we have yet to observe such neurons in any of our spinal cord stem cell progeny cultures. The culture conditions, e.g., presence of additional factors, may influence these expressions. In fact, the continued presence of EGF+bFGF likely underlies the low yield of differentiated cells, in comparison to that observed by Gritti and colleagues (1996). They suggest that removal of the mitogen allows for enhanced differentiation. It is noteworthy, however, that the addition of serum to plated adult forebrain spheres does not enhance neuronal differentiation, as was the case for embryonic stem cell progeny (Vescovi et al., 1993; Reynolds and Weiss, 1996), but seems to attenuate the process (C. Dunne and S. Weiss, unpublished observations). (3) To what extent do these findings in the mouse spinal cord extend to higher mammals? There are preliminary meeting reports of human equivalents to the embryonic stem cells that have been isolated and propagated in cell culture (Cattaneo et al., 1995). In addition, preliminary studies suggest that neural stem cells, which respond to EGF+bFGF, are present in the adult primate forebrain and spinal cord (S. Weiss, unpublished observations).

The presence of neural stem cells in the adult spinal cord and in the third and fourth ventricles raises some interesting practical considerations. In addition to putative roles in continued histogenesis of the adult CNS (to be determined), these cells may be amenable to modification and manipulation. Recently, Craig and co-workers (1996) demonstrated that infusion of EGF into the lateral ventricles resulted in enhanced proliferation of cells in the subependymal layer. Moreover, the cells migrated laterally and medially, in contrast to their normally circumscribed route along the rostral–caudal ventricular axis. Six to nine weeks after removal of the mitogen, new neurons and glia were observed in the striatal parenchyma. Thus, neural stem cells may be mobilized *in vivo*, and new neurons and glia can be delivered to sites within the mature CNS. It is reasonable to conclude from the results of the present study that such mobilizations may be possible in other regions of the mature CNS, such as the spinal cord. In particular, when speculating about their putative endogenous propensity to produce glia, one can envisage manipulating spinal cord stem cells in two different circumstances. The relative numbers of oligodendroglia and astrocytes would be critical in maintaining normal myelination in demyelinating conditions. In addition, the glial microenvironment may be modified after axotomy, to allow for enhanced regrowth. Additional studies aimed at understanding the endogenous *in vivo* properties of spinal cord neural stem cells, coupled with identification of the signaling molecules that direct the generation of specific lineages *in vitro*, will serve to guide the development of such strategies.

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Isolation and Transplantation of Multipotential Populations of Epidermal Growth Factor–Responsive, Neural Progenitor Cells From the Canine Brain

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Glial cell transplantation into myelin-deficient rodent models has resulted in myelination of axons and restoration of conduction velocity. The shaking (*sh*) pup canine myelin mutant is a useful model in which to test the ability to repair human myelin diseases, but as in humans, the canine donor supply for allografting is limited. A solution may be provided by self-renewing epidermal growth factor (EGF)-responsive multipotential neural progenitor cell populations ("neurospheres"). Nonadherent spherical clusters, similar in appearance to murine neurospheres, have been obtained from the brain of perinatal wildtype (*wt*) canine brain and expanded in vitro in the presence of EGF for at least 6 months. Most of the cells in these clusters express a nestin-related protein. Within 1–2 weeks after removal of EGF, cells from the clusters generate neurons, astrocytes, and both oligodendroglial progenitors and oligodendrocytes. Transplantation of lacZ-expressing *wt* neurospheres into the myelin-deficient (*md*) rat showed that a proportion of the cells differentiated into oligodendrocytes and produced myelin. In addition, cells from the neurosphere populations survived at least 6 weeks after grafting into a 14-day postnatal *sh* pup recipient and at least 2 weeks after grafting into an adult *sh* pup recipient. Thus, neurospheres provide a new source of allogeneic donor cells for transplantation studies in this mutant. J. Neurosci. Res. 50:862–871, 1997.

Key words: epidermal growth factor; myelin deficient; neural progenitor cells; glial cell transplantation

INTRODUCTION

Accumulating research in animal models raises hopes of using glial transplantation in therapy for human myelin diseases. Glia from a range of sources can myelinate axons in various recipient environments (reviewed in Duncan and Milward, 1995; Franklin and Blakemore, 1995; Duncan, 1996). Most research uses rodent recipients with inherited myelin disorders (reviewed in Duncan and Milward, 1995; Duncan, 1996) or with chemically induced demyelinating lesions (Blakemore and Crang, 1983; Blakemore et al., 1995), but there are obvious limitations to extending such studies to humans. Myelin diseases such as multiple sclerosis may develop over years, whereas most rodent myelin mutants die before adulthood. Glial cells grafted into rodents can migrate substantial distances (1–2 cm) but may need to travel much farther to reach surgically inaccessible lesions in humans. Mechanisms of differentiation, myelination, or remyelination may also differ between species. Most studies on human glia have not found close resemblances to rat oligodendroglial growth factor responses, antigenic profiles, or adult progenitor cells, although some similarities do exist (Kennedy et al., 1980; Dickson et al., 1985; Yong et al., 1988; Aloisi et al., 1992; Armstrong et al., 1992; Yong and Antel, 1993; Gogate et al., 1994; Satoh and Kim, 1994; Scolding et al., 1995).

The canine shaking (*sh*) pup model may help bridge the gap between rodents and humans. Like some rodent models and certain forms of the human Pelizaeus-Merzbacher and X-linked spastic paraplegia diseases, it arises from an exonic point mutation in the proteolipid protein gene (Nadon et al., 1990). The mutation arose spontaneously in Welsh springer spaniels and causes severe tremor from about 12 days of age after birth, followed by late onset convulsions (Griffiths et al., 1981; Duncan, 1995). The central nervous system (CNS) is

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severely hypomyelinated, with reduced numbers of mature oligodendrocytes (Duncan et al., 1983). Death normally occurs at about 3–4 months of age, but animals can live over 2 years with intensive rearing.

Xenografts of normal canine oligodendrocytes myelinate axons in myelin-deficient (*md*) rats (Archer et al., 1994), and allografting of canine glia into *sh* pup spinal cords shows that *sh* pup axons can be remyelinated (Archer et al., 1997). Allografting reduces immunologic complications but raises the problem of obtaining sufficient donor cells for transplantation, a common difficulty with species other than rodents. A solution may lie in the use of self-renewing, multipotential, growth factor–responsive neural progenitor cell populations, which grow in clusters dubbed "neurospheres" (Reynolds and Weiss, 1992, 1996; Reynolds et al., 1992). Cells in these populations generate neurons and astrocytes (Reynolds and Weiss, 1992, 1996; Reynolds et al., 1992) and oligodendroglial cells (Hammang et al., 1997; Reynolds and Weiss, 1996) in proportions that may be influenced by environmental manipulation. Murine neurospheres grafted into the *md* rat CNS generate cells that myelinate host axons (Hammang et al., 1994). Expandable multipotential populations of growth factor–responsive cells from the canine CNS could circumvent limitations of tissue availability for transplantation studies or in vitro analysis.

Although the presence of normal proteolipid protein or normal myelin production can distinguish normal from mutant oligodendrocytes after transplantation, both approaches are susceptible to technical or interpretational ambiguities. Recent glial transplantation has used *lac* Z expressing cells, obtained by stable transfection of rodent glial lines (Tontsch et al., 1994; Franklin et al., 1995, 1996) or from transgenic mice expressing *lacZ* under myelin protein promoters (Hammang et al., 1994; our unpublished data). Currently, no canine glial lines exist and neither approach can readily be applied in this species. Instead, we have tested the ability of the third generation PG13 retroviral vector system (Miller et al., 1991) to transduce *lacZ* expression in canine cells. Vector packaging in this system uses the Gibbon ape leukemia virus *env* protein, which includes both canine and human cells in its host range (Miller et al., 1991). A receptor, GLVR1, for this *env* protein is expressed abundantly in brain, with particularly high expression early in embryogenesis (Johann et al., 1992; Kavanaugh et al., 1994), making this system an ideal candidate for labeling canine neural progenitor cells.

We have obtained expandable multipotential populations of growth factor–responsive cells from the normal canine CNS and transduced these to express *lacZ* to provide labeled allogeneic donor cells for transplantation studies in *sh* pups or for xenografting into *md* rat recipients. We have also obtained similar populations from mutant *sh* pup brain, which should enable further study of the effects of the *sh* mutation on CNS cells.

MATERIALS AND METHODS

Preparation of "Neurospheres" From *sh* and *wt* Littermate Pups

Wildtype (*wt*) and *sh* pups were obtained from a colony at the University of Wisconsin. Donors were aged from embryonic day 40 to postnatal day 8. After euthanasia with barbiturate solution, brains were placed in artificial high-Ca^{2+}, low-Mg^{2+} cerebrospinal fluid solution (2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 124 mM NaCl, 5 mM KCl, 26 mM $NaHCO_3$, 10 mM D-glucose, pH 7.35) and the subventricular caudate nucleus, internal capsule, putamen/pallidum, and ventral mesencephalon separated and minced (<1 mm^3). Aliquots were dispersed mechanically by trituration or with enzymes (Reynolds and Weiss, 1992). All trituration was performed first with standard bore, then with fire-polished Pasteur pipettes, with the exception of trituration performed before transplant into *md* rats (see below), which used sequential passage through 20-, 23-, 25-, and 27-gauge needles. After filtration (35 μm nylon mesh; Small Parts, Miami Lakes, FL), suspensions were centrifuged (5°C, 5 min, 400*g*), dispersed in EGF^+ medium, comprised of 20 ng/ml mouse submaxillary gland EGF (Collaborative Research, Bedford, MA) in Dulbecco's Modified Eagle's Medium/F12 (1:1) with additives as given elsewhere (Reynolds et al., 1992), and plated in uncoated tissue culture flasks. To obtain adherent differentiated populations, cells were collected by centrifugation (5°C, 5 min, 400*g*), resuspended in EGF^- medium (the same base medium but with 1% fetal bovine serum replacing EGF) and plated on dishes coated with poly-L-ornithine (10 μg/ml).

Immunofluorescence

Rabbit polyclonal antibodies were used to detect nestin (Rabbit 130 from Dr. R. McKay, National Institute of Neurological Disorders and Stroke, Bethesda, MD) and 68 kD neurofilament protein (from Dr. P. Gambetti, Institute of Pathology, Case Western Reserve University School of Medicine, Cleveland OH). Monoclonal antibodies were Rat 401 anti-nestin (Developmental Studies Hybridoma Bank, Iowa City, IA), O4 and O1 (from Dr. M. Schachner), and Ranscht anti-galactocerebroside (GC) (Boehringer Mannheim, Indianapolis, IN). All other antibodies, conjugates, and nonspecific immunoglobulins were from Jackson Immunoresearch Laboratories (West Grove, PA). Negative controls were provided by omission of primary antibody or replacement with nonimmune rabbit serum or nonspecific mouse immunoglobulin. Secondary antibodies were the fluorescein-conjugates

goat anti-rabbit IgG and anti-mouse IgG, rhodamine-conjugated goat anti-mouse IgM, and biotin-SP-conjugated donkey anti-rabbit IgG and 7-amino-4-methylcoumarin-3-acetate-conjugated streptavidin. Cells were fixed with 4% paraformaldehyde (10 min, room temperature). Antibodies were diluted in 5% normal goat serum in phosphate buffered saline (with 0.1% Triton X-100 for antibodies to nestin). Aside from anti-GFAP (4°C, overnight), antibodies were incubated 1–2 hr at room temperature, followed by at least three buffer washes. After surface antigen labeling (O4, O1, Ranscht), samples were incubated in 5% glacial acetic acid:95% ethanol (v:v) for 10 min at −20°C and nestin, neurofilament, and glial fibrillary acidic protein (GFAP) antibodies used as above. Staining for combinations of O4, Ranscht and GFAP was as described elsewhere (Armstrong et al., 1992). Samples were mounted in CitiFluor (UKC Chemical Laboratory, Canterbury, UK) containing bisbenzimide H33342 fluorochrome (Calbiochem, La Jolla, CA).

Transduction of *lacZ* Expression in Canine Neurospheres

The PG13 retroviral packaging system, provided by Dr. M. Eiden, National Institute of Mental Health (Bethesda, MD), was maintained and used as previously described (Miller et al., 1991, 1993). Media conditioned for 6 days with packaged, defective, retroviral vector was harvested, filtered (0.45 μm), and used directly or stored at −70°C. Activity was confirmed using the Rat2 fibroblast line (Miller et al., 1991, 1993). Titers were in the order of 10^5 colony forming units per milliliter. Neurospheres were passaged (with trituration) into media consisting of EGF^+ medium:PG13-conditioned medium (1:1), with polybrene (4 μg/ml). After 20–24-hr incubation, cells were pelleted (5°C, 5 min, 400*g*), resuspended in EGF^+ medium, and collected 24–48 hr later by centrifugation (5°C, 5 min, 400*g*) for transplantation (below) or plating in EGF^- medium on poly-L-ornithine coated dishes for immunocytochemistry as above or 5-bromo-4-chloro-3-indolyl β-D-galactosidase (X-gal) staining (Tontsch et al., 1994).

Transplantation of Canine Neurospheres

After centrifugation as above, cells were resuspended in EGF^+ medium and either pipette triturated, followed by removal of undissociated spheres by nylon mesh filtration (35 μm), or triturated with needles as above. After Trypan Blue viability assessment and counting, cells were pelleted as above, resuspended at 25,000–75,000 cells/μl in Ca/Mg-free Hank's buffered saline solution with 0.01% bovine serum albumin and placed on ice until transplantation into *sh* pups.

Transplantation of Canine Neurospheres Into *sh* Pup Recipients

Recipient pups (aged either 14 days or 7 months) were premedicated with analgesics and sedatives, induced with an ultra-short-acting barbiturate, then intubated and maintained with isoflurane and oxygen. Dorsal laminectomy was performed at thoracic and lumbar sites T13–L1, L1–L2, and L2–L3. Using a surgical microscope, a durotomy was carried out at each laminectomy site. A glass micropipette (30-μm bore) was inserted into the spinal cord and 2–4 μl of cell suspension slowly injected over a 1-minute period by using a micromanipulator and Hamilton syringe. Respiration was controlled with the neuromuscular blocking agent succinylcholine during the injection to minimize spine movement. Injection sites were marked with sterile charcoal, a fat graft placed in each laminectomy defect and muscle, subcutaneous tissue and skin reapposed. Two to six weeks after injection, pups were anesthetized, fixative-perfused, and stained for X-gal, then transverse slices were Epon embedded for 1-μm sectioning and staining with *p*-phenylenediamine (Tontsch et al., 1994).

Transplantation of Canine Neurospheres Into *md* Rat Recipients

Cells were exposed three times to retroviral vector-containing medium that had been stored at −70°C (one freeze-thaw cycle). This boosted the transduction efficiency obtained with stored media. Initial overnight vector exposure, centrifugation, and resuspension in EGF^+ medium were as described above; after 48 hr, cells were again passaged with trituration into EGF^+ medium: PG13-conditioned medium (1:1) with polybrene (4 μg/ml), incubated 4 hr, then centrifuged and resuspended as before. This was repeated 72 hr later. Cells were passaged with trituration 48 hr later, and after a further 24 hr, triturated as above, except that the last trituration used 25½- and 27-gauge needles sequentially, and placed on ice for transplantation. The *md* rat recipients were from a colony at the University of Wisconsin. Transplantation into *md* rat spinal cord consisted of either one or two injections, the latter 1 mm apart, each of 1 μl at 25–50,000 cells/μl at the thoracic–lumbar T13–L1 junction, using published protocols (Hammang et al., 1994). Recipients were treated daily with Cyclosporine A (10 mg/kg body weight). Conditions of final anesthesia, tissue fixation, processing for X-gal revelation, Epon embedding, 1-μm transverse sectioning, and *p*-phenylenediamine staining have been described (Hammang et al., 1994; Tontsch et al., 1994).

RESULTS

Preparation of Neurospheres From *sh* Pup and *wt* Littermate Pups

We tested various tissue dissociation techniques on several regions from embryonic or postnatal *wt* canine brains. Donors were aged between embryonic day 40 to postnatal day 2 (canine gestation is about 63 days). Cells from embryos multiply more rapidly and have been expanded in vitro at least 6 months.

Neurosphere-like clusters were derived from each of the subventricular caudate nucleus, internal capsule, putamen/pallidum, and ventral mesencephalon. For all protocols, the putamen/pallidum was consistently the poorest source, both in cluster yields and in long-term sustainability of cultures. Subventricular and ventral mesencephalon regions were the richest sources of neurospheres from embryonic and postnatal pups and the most amenable to sustained in vitro expansion, as is also the case in both embryonic and adult mice (Reynolds and Weiss, 1992; Reynolds et al., 1992; Hammang et al., 1994, 1997). Separate canine preparations were subsequently made from (1) ventral mesencephalon and (2) "striatum," comprised of pooled caudate nucleus and the adjacent portion of the inner capsule.

Rodent neurospheres are obtained from CNS by trituration alone or after enzyme treatment (Reynolds and Weiss, 1992; Reynolds et al., 1992). Trituration alone routinely generated neurospheres from canine CNS, but enzyme treatment failed to give useful yields from some pups and subsequently was not used. Moreover, compared with rodent neurospheres, even after isolation, canine neurospheres were harder to dissociate during subsequent passaging or for differentiation studies. Even forceful trituration failed to disperse all clusters and, gauged by Trypan Blue exclusion, damaged as many as 80% of cells (data not shown).

In the murine neurosphere system, when cells are first isolated from the CNS there is a period of cell death over the first 5 days in vitro, followed by progenitor division leading to sphere formation (Reynolds et al., 1992). This was not observed in the canine system. Instead, adherent spherical clusters of refractile cells of healthy appearance were first detectable at 4 hr postplating and were readily apparent by 12 hr in both striatal and ventral mesencephalic cultures. As preparations were filtered before plating, these clusters may arise from cell aggregation. Nonadherent spherical clusters containing four or more refractile cells were observed within 12–72 hr postplating in essentially all cultures (Fig. 1A.). Debris and unhealthy or dead-looking cells were typically present during the first 14 days, but apparently healthy spheres of increasing size continued to be observed throughout this time. Dispersion of large clusters during passaging (see Methods and previous paragraph) yielded both small clusters and individual cells, with subsequent sphere growth allowing expansion at a split ratio of 1:2 every 2–4 weeks for at least 6 months

After expansion by passaging between two to nine times in the presence of EGF, adherent differentiated populations were obtained by partially dissociating neurospheres by trituration and plating onto poly-L-ornithine substrata in EGF medium. (As noted above, spheres could not be completely dissociated without excessive cell destruction.) Cells were fixed 1 hr to 28 days later. By 1 hr postplating, spheres had attached to the substratum. By 24 hr, emergent cells began to form monolayer "halos" around spheres.

Rat401 monoclonal and rabbit polyclonal 130 antibodies were used to reveal expression of nestin, a neural progenitor marker, in striatal or ventral mesencephalon-derived neurospheres. At 1, 4, and 24 hr postplating, fluorescence was above background levels in most cells (Fig. 1B), although labeling intensity with either antibody was low compared with staining seen with Rat401 in murine systems (Hammang et al., 1994; our unpublished observations), suggesting epitopic differences between canine and rodent nestin species. Both cells in clusters and some cells in the monolayer expressed nestin, but by 24 hr postplating many cells in the monolayer were apparently unlabeled (Fig. 1B) and no nestin staining was detectable after 10 days in vitro (not shown). Patterns of nestin staining were similar in cells originally derived from different CNS regions.

Monoclonal antibody to the 68 kD NF protein detected cells with morphologies resembling neurons or immature neuronal cells at 25 days postplating in both striatal and ventral mesencephalon-derived cultures (Fig. 1C). As revealed by this antibody, many of these cells had long, very fine processes. No cells expressing the 68-kD NF protein were detected at earlier times. Cells expressing GFAP, some with stellate morphology, were detected at all times examined from 7 days postplating onward in both striatal and ventral mesencephalon-derived cultures (Fig. 1D).

Markers used with or without concomitant GFAP staining to identify oligodendrocyte-type 2 astrocyte lineage cells were the O4 and Ranscht antigens (Sommer and Schachner, 1981; Ranscht et al., 1982). Process-bearing cells expressing O4 were detected within and surrounding spheres at all times examined from 7 days postplating onward in both striatal and ventral mesencephalon-derived cultures (Fig. 1E). Some, but not all, of these O4+ cells also expressed GFAP. Neither cells of stellate morphology nor process-bearing cells with morphologies typical of O4+ cells were observed in freshly

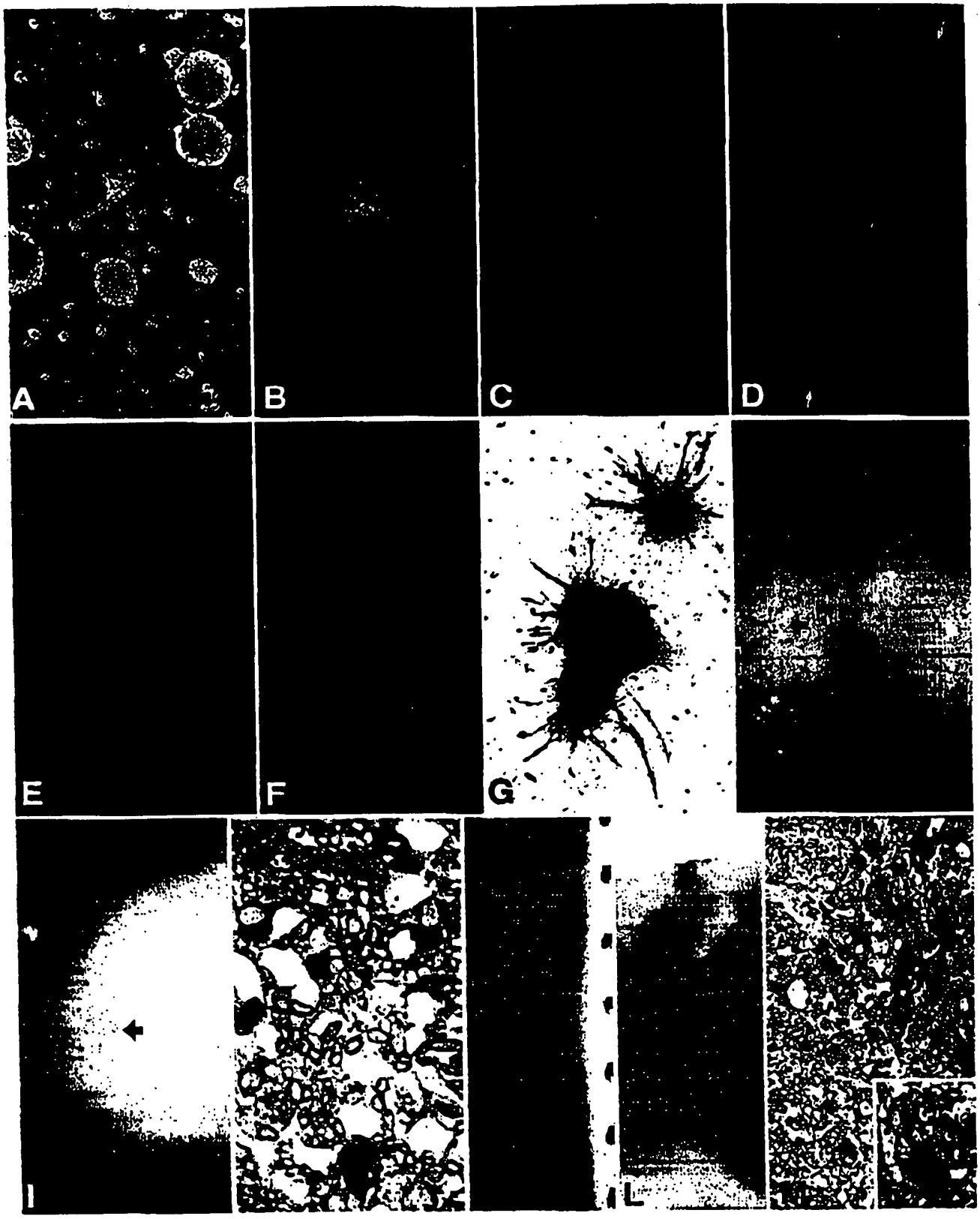

Figure 1.

plated neurosphere cultures nor in the surrounding halos of cells migrating out of the neurospheres in the first 24 hours after plating. Ranscht antigen expression was not detected at either 7 or 10 days postplating in any cultures but was observed at 12 days postplating in all cultures in a very small percentage of $O4^+$ cells (estimated to be $\ll 1\%$). These typically had morphologies classically associated with oligodendrocytes, with many highly branched processes (Fig. 1F). Such morphologies were not observed in freshly plated neurosphere cultures nor in the surrounding halos of cells migrating out of the neurospheres in the first 24 hr after plating from either earlier or later passages. These morphologic observations and patterns of marker expression, in particular the delayed and separate appearances of the Ranscht antigen and the 68-kD NF protein, provide direct evidence that differentiation along CNS lineages is occurring in this system.

The PG13 packaging system uses the Gibbon ape leukemia virus *env* protein (Miller et al., 1991). The retroviral vector we used in this system is based on the plasmid pLXSN and contains *lacZ* under the SV40 promoter (Miller et al., 1993). This vector/packaging combination transduced *lacZ* expression in a substantial proportion of cells in the canine neurosphere preparations generated from perinatal pups (Fig. 1G). We first used allografting into *sh* pups to address the issue of whether neurosphere-derived cells can survive in the adult *sh* pup CNS environment. Adult *sh* pups are extremely rare, and although the lifespan of *sh* pups can be increased to more than 2 years with intensive rearing, individual adults may nonetheless die at any time after 3–4 months even without the added risks arising from surgery. For these reasons, in this initial study, survival in an adult animal was examined in the short term, and a postnatal animal was used to examine survival in the longer term. Fixation and processing conditions for combined *lacZ* and myelin detection have been refined in numerous rat studies and are not optimal with respect to tissue preservation in the larger canine system, where animal rarity restricts optimization. Thus, the myelinating capacity of neurosphere populations was examined by xenografting into *md* rats, which also provides more stringent environmental conditions than allografting. Aliquots of populations destined for allografts or xenografts were plated under differentiating conditions, as above and in Methods, for parallel examination of in vitro differentiation and *lacZ* expression. The appearance of differentiation markers in these cultures followed the same time course as was described above.

Populations of proliferative canine neurospheres, originally derived from striatum of embryonic day 40–50 normal pups, were transduced to express *lac* Z and transplanted into the spinal cords of a postnatal 14 day and an adult (7 months) *sh* pup. (Donor cells had undergone three and nine passages in vitro, respectively, before transplant.) The postnatal pup was killed at 6 weeks and the adult pup at 2 weeks posttransplant. After X-gal staining, transverse sections of the spinal cord in the regions of the thoracic and lumbar injection sites (T13–L1, L1–L2, and L2–L3) revealed distinct clusters of blue patches in each of the postnatal and adult animals not only at the site of implantation in the dorsal column but also in the lateral or ventral columns and gray matter (Fig. 1H, I), suggesting that migration of implanted cells

Fig. 1. Generation and transplantation of EGF-responsive canine neural progenitor populations. A: Preparation from the striatum of a normal E45–50 pup at 48 hr after isolation from the CNS, showing spherical clusters resembling neurospheres containing refractile cells of healthy appearance. B: Nestin immunoreactivity (green) occurs in cells within and around spheres in normal E50 canine striatal neurosphere preparations 24 hr after plating in EGF medium. Some surrounding cells, visualized by nuclear labeling with Hoechst bisbenzimide H33342 (blue), do not express nestin. C: Cells from normal E50 canine striatal neurospheres 25 days after plating in EGF medium, labeled for the 68-kD neurofilament protein (green) and Hoechst bisbenzimide H33342 (blue). D: Cells from normal E50 canine striatal neurospheres with stellate and other morphologies expressing GFAP (blue) 7 days after plating in EGF medium. Nuclei are also labeled blue with Hoechst 33342, which does not label cell processes, allowing GFAP-negative cells to be visualized (arrows). E: Expression of O4 antigen (red) in cells from normal E50 canine ventral mesencephalon neurospheres 12 days after plating in EGF^- medium. F: Occasional O4-positive cells also react with the Ranscht anti-galactocerebroside antibody at this time. G: Expression of *lacZ* in normal canine neurospheres after transduction using the PG13 retroviral vector system. H: Transverse section of the spinal cord of a 2-month *sh* pup, 6 weeks after transplantation of *lac* Z-labeled *wt* canine neurospheres at 14 days after birth. Blue clusters are visible in several regions of the cord (arrows). I: Transverse section of the spinal cord of a 7–8-month *sh* pup, 2 weeks after transplantation of *lacZ*-labeled *wt* canine neurospheres. A blue cluster is visible in the lateral column of the spinal cord (arrow). J: Microscopy of transverse sections shows graft cells apparently integrated normally into the adult *sh* pup cytoarchitecture (arrows). Vacuolation in this section is due to the method of fixation and not the transplanted cells. The section is counterstained with paraphenylenediamine. K: Blue clusters are visible for several millimeters along the dorsal midline of the spinal cord of an *md* rat that received *lacZ*-labeled canine neurospheres 1 week after birth (1-mm markers on right). L: The same spinal cord shown in K, showing blue labeling throughout the dorsal column at the site of injection. M: In a 1-μm section from the spinal cord shown in L, scattered myelinated fibers (more than seen in nontransplanted *md* rats) are present. Details of a normal oligodendrocyte with a long cytoplasmic process extending to a myelinated axon (arrow) are shown in the inset. Such cells are not normally seen in areas outside of the transplant.

may have occurred. Our previous studies have shown that there is an acute passive dispersion of cells only along the dorsal column, up to 8 mm from the site of transplantation (Lipsitz et al., 1995). Similar studies with Hoechst labeled cells have also shown a localization of transplanted cells immediately after transplant to the dorsal columns (Zhang and Duncan, unpublished data). The fixation used failed to preserve structures optimally in the adult mutant, but nonetheless light microscopy of tissue sections clearly demonstrated *lacZ*-expressing cells in association with areas containing myelin (Fig. 1J). These cells appeared to have integrated into the surrounding cytoarchitecture, with no evidence of rejection.

Xenografts into *md* rat recipients were used to obtain further evidence for the ability of neurosphere-derived cells to myelinate host axons in a myelin-deficient environment. To facilitate routine studies in these rats, a triple exposure protocol was used for successful transduction of *lacZ* expression by using media conditioned with packaged, defective, retroviral vector that had been stored frozen before use (Methods). Canine neurospheres were transduced to express *lacZ* and implanted into the dorsal columns of spinal cords of 7-day postnatal *md* rats at the thoracic–lumbar (T13–L1) junction (Methods). At 11 days posttransplant, low-power light microscopy revealed blue X-gal reaction product spread up to 6 mm along the dorsal midline of at least three transplant recipients in each of two separate experiments (Fig. 1K). This typically appeared as clusters of small blue patches or dots, rather than the more homogeneously distributed streak of blue-labeled cells often seen after glial cell transplantation (Tontsch et al., 1994). Transverse sections (1 μm) of recipient spinal cords revealed grafted cells in the dorsal columns in areas containing myelinated axons (Fig. 1L) and, strikingly, a grafted cell with a long cytoplasmic process in contact with a myelinated axon (Fig. 1M). By electron microscopy (EM), the myelin was found to have a normal intraperiod line that is lacking in host myelin.

DISCUSSION

We have obtained cell populations from *wt* canine perinatal striatum and ventral mesencephalon that closely resemble murine EGF-responsive multipotential neural progenitor cell populations (neurospheres). The clusters of canine cells are similar in appearance to murine spheres and can be expanded with passaging for at least 6 months in vitro in the presence of EGF. On removal of EGF, cells within the clusters generate neurons, astrocytes, and oligodendroglia. Cells from these cultures can survive at least 6 weeks in postnatal and at least 2 weeks in adult *sh* pup recipients. This is the first time neurosphere transplant survival has been demonstrated in the adult CNS. Neurospheres thus provide a new source of allogeneic donor cells for transplantation studies in this mutant. Moreover, grafting of *wt* neurospheres into *md* recipient spinal cords resulted in the production of apparently normal myelin by graft-derived cells, showing that these cells are able to myelinate a myelin-deficient host. The ability of neurosphere-derived cells to myelinate under xenograft conditions is encouraging for future studies involving human neurospheres, which must first be tested by xenografting. In addition, the PG13 retroviral vector packaging system, based on the Gibbon ape leukemia virus *env* protein, provides a new means of labeling donor cells. With a host range also including humans and rodents, this system is likely to be useful for transplantation studies in various species.

Our observations of neurospheres within a few hours of the initial preparation of cultures from the CNS suggest rapid extensive reaggregation of cells. Early "spheres" are unlikely to arise from proliferation of single progenitors as canine neurospheres grow slowly compared with murine neurospheres maintained in identical media (our unpublished observations). Human fetal neurosphere preparations also grow very slowly compared with murine preparations and may require different combinations of factors for optimal growth (B. Reynolds, personal communication). Faster growth apparently occurred in preparations obtained from embryonic, as opposed to postnatal, canine CNS. Because initial yields from postnatal pups were in general lower than those obtained from embryos, the relative success of the latter may reflect either or both developmental differences in the proliferative capacities of progenitors or growth-enhancing autocrine effects, which may occur in higher density cultures.

Removal of EGF resulted in the sequential appearance, over a period of 3–4 weeks, of cells with distinctive differentiated morphologies expressing markers of mature astrocytes, oligodendrocytes, and neurons, which were not detected in freshly plated neurosphere preparations. Thus canine neurosphere-derived cells differentiate along at least two lineages, although we could not verify that these neurospheres do, in fact, contain multipotential cells rather than subpopulations of lineage-committed, unipotential progenitor cells, because the slow growth rate of these cells has prevented clonal analysis to date. Clonal analysis has confirmed the existence of self-replicating neural stem cells in analogous EGF-responsive murine neural progenitor populations (Reynolds and Weiss, 1996), and the long-term expandability of canine populations is indirect evidence that such cells may be present. However, whether or not stem cells are present, as the first expandable differentiating system established from canine brain, neurospheres provide a means to study at least three of the major CNS cell species, which has not

previously been available in the dog and which should facilitate studies both of normal canine and also of *sh* pup CNS.

Although secondary to the aims of the present study, we have had preliminary success in generating neurospheres from *sh* pup mutant embryos (data not shown), providing a new in vitro system for characterizing the effects of the mutation. The developmentally regulated alternative splicing pattern of the myelin proteolipid protein gene is altered in the *sh* mutant, suggesting delayed mutant oligodendrocyte maturation (Nadon et al., 1990). Proteolipid protein gene expression may also have functions in CNS development before myelinogenesis. The ability to generate neurospheres that are similar in many respects to those derived from *wt* brain should facilitate studies on differentiation of *sh* pup oligodendrocytes. Such *sh* pup neurospheres could also be used as a target in gene transfer studies. In addition, our success in expanding neurospheres from the *sh* pup mutant suggests that this approach could extend the range of models amenable to study and may be applicable to very rare mutants, in which limited availability of affected animals has restricted characterization to date.

Although expansion of the canine populations is slow compared with the murine case, the scarcity of source tissue in the canine system makes these cells a valuable alternative to primary culture. Analysis of growth factor effects in the canine system may permit faster expansion of these cells in vitro. Basic fibroblast growth factor (bFGF) stimulates proliferation of some rodent neural progenitor populations (Lillien and Cepko, 1992; Richards et al., 1992; Ray and Gage, 1994; Kilpatrick and Bartlett, 1995; Vescovi et al., 1993) and has been implicated in oligodendroglial proliferations and inhibition of differentiation or population reversion to more immature phenotypes in both rat (Bogler et al., 1990; McKinnon et al., 1990) and human models (Armstrong et al., 1992; Gogate et al., 1994). It could well have similar effects in the canine system, in which bFGF (5–100 ng/ml) may increase proliferation in canine oligodendroglial-enriched cultures (Hoffman and Duncan, 1995). Sequential growth factor mixtures could enhance first proliferation then differentiation of canine neural progenitor cells.

Myelin deficiency may activate compensatory mechanisms. Even when these are insufficient to override effects of endogenous myelin gene mutations, the environment may nonetheless express factors favoring oligodendrogenesis and myelinogenesis by transplanted cells. Neurospheres generated myelinogenic oligodendrocytes when transplanted into the *md* rat, and furthermore, grafted neurosphere-derived cells survived not only in the neonatal but also in the adult *sh* pup. Thus the adult canine environment is able to support the survival of perinatal neural progenitor cells, at least in the short term. This is consistent with the existence of neural progenitor cells in adult rodent CNS (Reynolds and Weiss, 1992; Richards et al., 1992). The number of myelinated axons observed after xenografting of canine neurospheres into the *md* rat was considerably more than is seen in nontransplanted *md* rats, although less has been seen with allografts of murine neurospheres, which myelinate large areas of the *md* cord (Hammang et al., in press). In view of the in vitro differences in expansion rates discussed above, canine neurospheres may also take longer to expand and mature in vivo than murine neurospheres, even though the presence of apparently myelinating transplant-derived oligodendrocytes after only 11 days in vivo suggest canine oligodendrocytes differentiate more rapidly in vivo than in vitro. This system should be valuable for future study of survival, growth, and myelinating ability of grafted cells, particularly in adult recipients, with ultimate applications in humans.

Unlike some more differentiated CNS cell species, neural progenitor cells may not express both classic histocompatibility molecules and may thus, at least initially, provoke a less intense host immune reaction (Bartlett et al., 1990). Although null mutant studies suggest peripheral transplant rejection can occur in the absence of both class I and II molecules, as far as we are aware this has yet to be established for the CNS, which is relatively immunologically privileged. Neurospheres also offer the prospect of generating mixed populations of neural cell species in a controllable manner. Because both astrocytes (Raff et al., 1985; Richardson et al., 1988) and neurons (Barres and Raff, 1993; Hardy and Reynolds, 1993) have been implicated in oligodendroglial migration and differentiation and may also influence myelination, this could benefit transplant-derived remyelination. As well as advantages of manipulability and in vitro expandability, these cells provide a multipotential, proliferation-competent pool from which differentiated populations may arise in response to environmental cues. Implantation of small donor populations could ultimately assist in maintaining lifelong reservoirs from which CNS cell populations may be renewed through endogenous or externally manipulated environmental signals.

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Myelination Following Transplantation of EGF-Responsive Neural Stem Cells into a Myelin-Deficient Environment

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Epidermal growth factor (EGF)-responsive stem cells have been identified in the murine central nervous system. These cells can be isolated from the brain and maintained in an undifferentiated state *in vitro* in the presence of EGF. After removing EGF, the cells cease mitosis and can be induced to differentiate into neurons, astrocytes, and oligodendrocytes. We demonstrate that when the undifferentiated stem cells (nestin-positive) are injected into the myelin-deficient rat spinal cord, they respond to cues within the mutant CNS and differentiate into myelinating oligodendrocytes, in contrast to their behavior *in vitro*, where they mainly form astrocytes. The cells provide a valuable model system for the study of the development of early oligodendrocytes from multipotent neural stem cells. Because these cells are influenced to divide using growth factors, rather than oncogenes, and because they appear to make appropriate lineage decisions when transplanted into a mutant environment, they may provide an excellent source of cells for a variety of future therapies using cellular transplantation. © 1997 Academic Press

INTRODUCTION

The mammalian central nervous system is a highly ordered and complex structure composed of a variety of neurons and glial cells. The development and maintenance of these cell types is dependent upon and influenced by a number of intrinsic genetic factors as well as environmental signals. Such signals may be provided by cell to cell contact and/or the secretion of neurotransmitters and neurotrophic factors. During normal development, neural cells are formed in a temporal sequence that is governed by these intrinsic and environmental signals (7). In some situations, the exact spectrum of signals necessary to specify a particular cell fate may be expressed only at certain times during CNS development. The signals necessary to influence oligodendrocyte development, however, appear to be maintained after the period of time during which the majority of oligodendrocytes develop and form myelin as evidenced by transplantation studies in several animal models of both dysmyelinating and demyelinating diseases (12). A number of experiments have been performed in which precursor cells isolated either from primary sources, or oncogene-immortalized glial precursors, have been transplanted into the CNS (14). In these experiments, varying, but significant degrees of myelination have been achieved, suggesting that given an appropriate source of transplantable cells or tissues, one could eventually attempt the replacement of CNS myelin in humans (12).

A population of epidermal growth factor (EGF)-responsive multipotential stem cells derived from the embryonic and adult murine brain (27, 26) or the fetal human brain (25, 6, 30) have been isolated and characterized *in vitro*. In the presence of EGF, these cells can be continuously propagated with weekly passaging for at least 1 year, maintaining a stem cell-like phenotype. Using an *in vitro* characterization, we define the cells as stem cells based on the multipotential characteristics and by their expression of the intermediate filament marker, nestin (19). By removing the EGF and with the addition of a small amount of fetal bovine serum, the stem cells can be induced to differentiate into neurons, astrocytes, and oligodendrocytes when plated on a polyornithine-treated substrate (17). *In vitro*, the relative numbers of differentiated cells can be directly influenced by the addition of neurotrophic factors and cytokines (1). Specifically, the addition of CNTF, LIF, or oncostatin M leads to a significant increase in the numbers of oligodendrocytes (28).

In the present studies we sought to determine whether these EGF-responsive neural stem cells (hereafter termed neurospheres), which are clearly multipotential *in vitro*, were capable of responding to cues within a dysmyelinated environment of a mutant animal to form mature, functioning cells of the appropriate type. The CNS myelin mutant, the myelin-deficient (*md*) rat, is characterized by a failure of development and early death of oligodendrocytes (18, 13). It has

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previously been shown that the *md* CNS is virtually devoid of myelin and that its axons are capable of recruiting and supporting transplanted myelinating cells (10, 29, 31). Therefore, the stem cells were transplanted into the spinal cord of the *md* rat to determine whether they were capable of differentiating *in vivo* into oligodendrocytes and myelinating axons. Undifferentiated neurospheres were mechanically dissociated to yield a single-cell suspension and were injected directly into the thoracolumbar region of 6- to 8-day-old *md* rats. Our results indicate that when transplanted into the *md* CNS, these multipotent stem cells preferentially form oligodendrocytes leading to extensive myelination. Light and electron microscopic evaluation of the regions of myelination reveal no gliotic response nor any evidence of aberrant or inappropriate neuronal differentiation in any of the animals examined. This is the first study to demonstrate that multipotential stem cells, which have not been transformed or immortalized with oncogenes, and which possess no clear glial or neuronal phenotype prior to transplantation, can respond to cues within the CNS and form myelin *in vivo*. The cell line characteristics, including a self-renewing capacity under the control of growth factors, which are easily removed, and a multipotential nature, should provide a more suitable system with which to study CNS development and oligodendrocyte specification (15). Furthermore, because these stem cells are primary in nature and can be isolated from human tissue, they may provide a suitable source of transplantable material for CNS repair in humans.

EXPERIMENTAL METHODS

Preparation and Maintenance of Neural Stem Cell Cultures

Using sterile technique, the striata and ventral mesencephala from litters of E14–15 Sprague–Dawley rats and BalbC mice were dissected and separately pooled in L-15 dissection buffer and held on ice. The L-15 was removed and the dissected tissue was resuspended in a defined, serum-free medium containing 20 ng/ml EGF (complete EGF medium) (27), and vigorously triturated 10–20 times with a fire-polished Pasteur pipet. Striatal and mesencephalic cells were plated at 100,000 cells per milliliter in T25 flasks and maintained in the complete EGF medium. The neural stem cells grew in clusters (spheres) that tended to be free-floating in the flasks. For passaging, the clusters were harvested, gently centrifuged at 200*g*, and mechanically dissociated in the EGF-containing medium by trituration with a fire-polished Pasteur pipet. Following the trituration, the single cell suspension was diluted in the EGF medium and replated at approximately 10^6 cells in T75 flasks. During the course of these experiments, the cells were passaged once per week for up to 25 passages.

Immunocytochemical and Immunohistochemical Methods

The stem cells can be induced to differentiate into oligodendrocytes, astrocytes, and neurons by altering culture conditions. The free-floating stem cell clusters that were continually passaged in EGF were gently centrifuged, resuspended in the same base, defined medium (with no EGF) with 1% fetal bovine serum, and plated on polyornithine-treated glass coverslips. The clusters attached firmly to the glass, and the cells slowed or stopped dividing and differentiated. One to 14 days postplating, cells on coverslips were incubated unfixed, for 30 min at room temperature with the primary antibodies against O1, O4, GalC, and A2B5 (supernatants) diluted in minimal essential medium with 5% normal goat serum and 25 m*M* Hepes buffer, pH 7.3 (MEM-Hepes, NGS). Following the primary antibodies, the coverslips were gently washed five times in MEM-Hepes and incubated for 30 min at room temperature in fluorescein- or rhodamine-conjugated secondary antibodies (Sigma) diluted in MEM-Hepes, NGS, as recommended by the manufacturer. The coverslips were then washed five times in MEM-Hepes and fixed with acid alcohol (5% glacial acetic acid/95% ethanol) at −20°C. The coverslips were then washed five times with MEM-Hepes, and either mounted and examined using fluorescence microscopy or immunoreacted with rabbit polyclonal antisera raised against GFAP (Dako), nestin (R. McKay), MBP (Serotech), or PLP (Serotech). When subjected to a second round of immunolabeling, the coverslips were incubated first for 1 h with 5% normal goat serum (NGS) in 0.1 *M* phosphate buffer with 0.9% NaCl at pH 7.4 (PBS) and then incubated in rabbit primary antibodies diluted in NGS for 1–2 h at room temperature. The coverslips were washed three times with PBS and then incubated with the appropriate secondary antibody conjugates diluted in NGS, washed again with PBS, and then finally mounted on glass microscope slides with Citifluor antifadent mounting medium and examined using a fluorescence microscope. In cases where they were not incubated first with the monoclonal antibody supernatants, the coverslips were fixed for 20 min with 4% paraformaldehyde in PBS (pH 7.4), washed with PBS, permeabilized with 100% ethanol, washed again with PBS, and incubated with 5% NGS in PBS for 1 h. The primary antibodies and secondary antibody conjugates were applied as outlined above.

For glial fibrillary acid protein (GFAP) immunostaining, 1-μm sections were sealed onto glass slides and the Epon was etched with a saturated solution of sodium ethoxide diluted 1:1 with 100% ethanol. Sections were then incubated with rabbit anti-cow GFAP (Dako, Carpinteria, CA). The primary antibody was detected using the Vectastain Elite kit (Vector Laboratories, Burlingame, CA), which includes a biotinylated goat

anti-rabbit IgG secondary antibody, in conjunction with diaminobenzidine and osmium tetroxide.

Animals and Surgical Procedures

The neural stem cells were continuously maintained in EGF-containing medium for up to 25 passages. For implantation, nestin-positive neurospheres (no mature oligodendrocytes) were collected, gently centrifuged at 200*g*, and triturated into a single cell suspension in HBSS with 0.1% BSA (Sigma). This suspension was concentrated to 50,000 cells/μl in the same HBSS/BSA medium prior to injection. Litters of *md* rats and their control littermates at 6–8 days postnatal were anesthetized with halothane and a small longitudinal incision was made along the back, and a laminectomy was performed exposing the T13-L1 level of the spinal cord. A small cut was made in the dura mater to permit the entry of the glass micropipet. Approximately 1.0–1.5 μl of the stem cells in HBSS/BSA were then injected into the dorsal columns just lateral to the midline. The site of injection was marked using sterile charcoal powder. Following the injection, the incision was closed with sutures and the animals were gently warmed and allowed to recover from the anesthesia. The animals were then returned to the dams and allowed to survive for approximately 2 weeks. Those animals transplanted with the mouse stem cells received Cyclosporin-A (daily) at a dose of 10 mg/kg IP for the duration of the experiment.

Histological Analysis, Autoradiography, and Electron Microscopy

At approximately 12–14 days after the stem cell transplants, the animals were prepared for sacrifice. Ninety minutes prior to sacrifice, the animals were injected with tritiated thymidine at a dose of 0.002 mCi/g. Following the 90 min, the animals were deeply anesthetized with pentobarbital and perfused via the left ventricle with a modified Karnovsky's aldehyde fixative. The spinal cord surrounding the transplant site was removed and 1-mm sections were processed and embedded for light and electron microscopy. For LM autoradiography, 1-μm Epon sections were heat sealed onto glass slides, dipped in NTB2 emulsion (Eastman Kodak, Rochester, NY), allowed to expose for 2 months while stored at 4°C, and developed with Kodak D19. The autoradiographs were counterstained with 1.0% alkaline toluidine blue. Nuclei overlain with at least four silver grains were considered to be labeled. For electron microscopy, Epon sections (70–90 nm) were cut with a diamond knife, placed on copper-rhodium mesh grids, contrasted with lead citrate and uranyl acetate, and photographed with a Philips 410 electron microscope.

RESULTS

CNS Stem Cells form Mature Oligodendrocytes In Vitro

Stem cell cultures were prepared from both BalbC mouse and Sprague–Dawley rat fetuses at approximately 15 days of gestation. Striata and ventral mesencephala were dissected separately from fetuses and mechanically dissociated and cultured in a serum-free, defined medium containing 20 ng/ml EGF (complete EGF medium—see Experimental Methods). Under these culture conditions, the stem cells continued to divide, forming relatively tight clusters which increased in size over the 1-week culture period (Fig. 1A). During the course of these experiments, separate striatal and mesencephalic cultures were passaged once per week for up to 25 passages using mechanical dissociation. The stem cells were periodically induced to differentiate to confirm that they maintained their multipotentiality *in vitro* during the later passages. The stem cells differentiated upon the removal of EGF, with the addition of 1% fetal bovine serum and plating on a polyornithine-treated substrate. For *in vitro* differentiation, the stem cells were either plated without dissociation as clusters or were mechanically dissociated to a single cell suspension and then plated on polyornithine-treated glass coverslips.

To identify the specific cell types after *in vitro* differentiation, the coverslips were immunoreacted with antibodies specific for oligodendrocyte precursors or mature oligodendrocytes, 1 to 7 days after inducing differentiation. By 1 day postplating, the clusters had firmly attached and were considerably flattened. The flat cells at the base of the attached clusters were initially intensely immunoreactive for nestin. By 3 days, however, the nestin immunoreactivity had been replaced by an increasing immunoreactivity to GFAP in the same cells, consistent with the differentiation of astrocytes. During the same period, a large number of cells with a bipolar morphology were evident, and most of these cells had migrated primarily to the periphery of the adherent clusters (Fig. 1B). At this 3-day time point, O4 and GalC immunoreactivity was clearly evident in the same population of bipolar cells, with the O4+ and GalC+ cells having the more complex processes, as did the bipolar cells evident during the initial 1–2 days postdifferentiation (Fig. 1C). At 5 to 7 days postplating, nestin and O4 immunoreactivity was virtually absent in all of the cells. A fraction (in the range of 0.1–1%) of the previously O4+ and GalC+ oligodendrocyte precursors, based on the peripheral position of the cells surrounding clusters, became immunoreactive to O1, MBP, and PLP, and possessed a distinct oligodendrocyte morphology (Figs. 1D–1F).

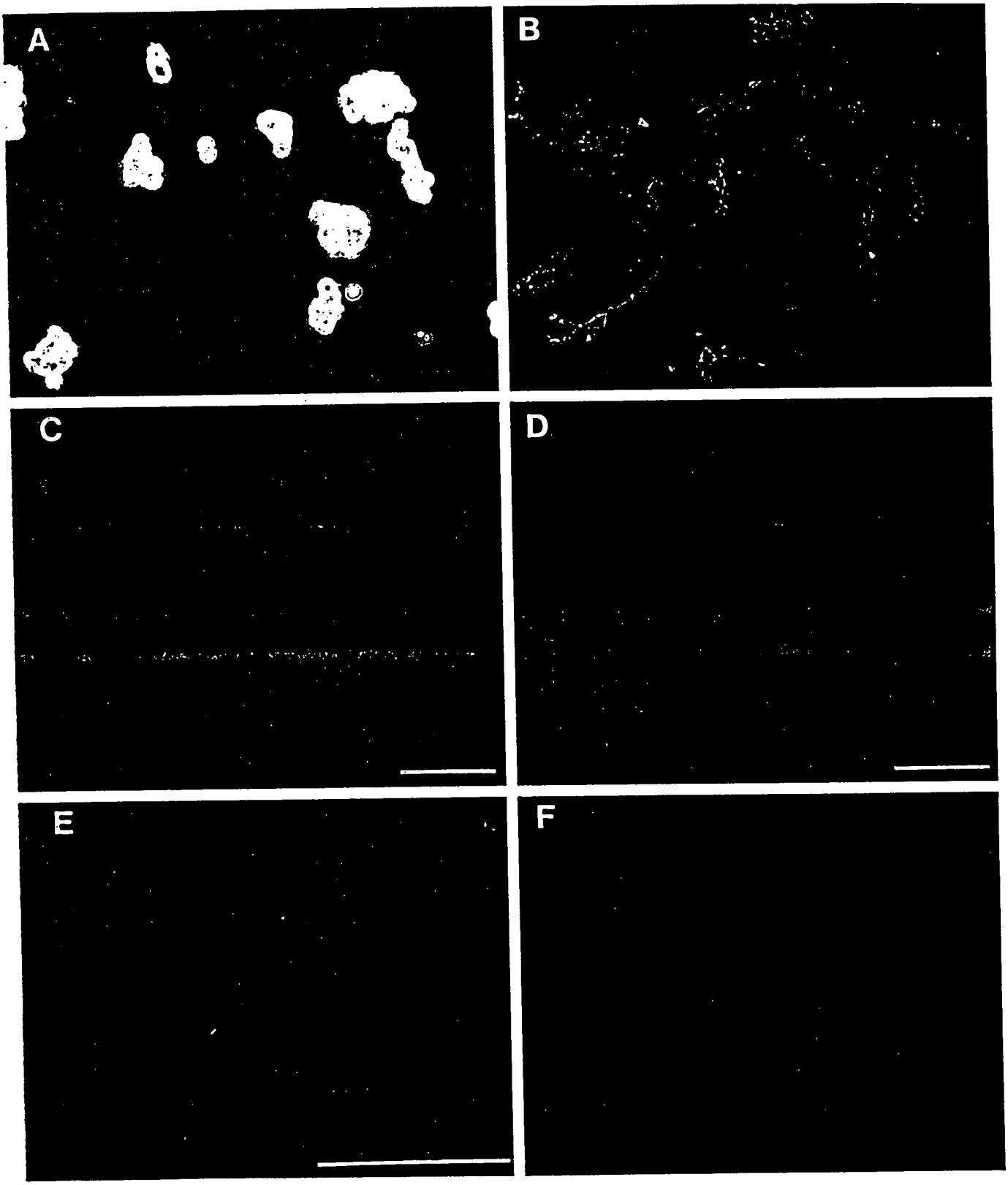
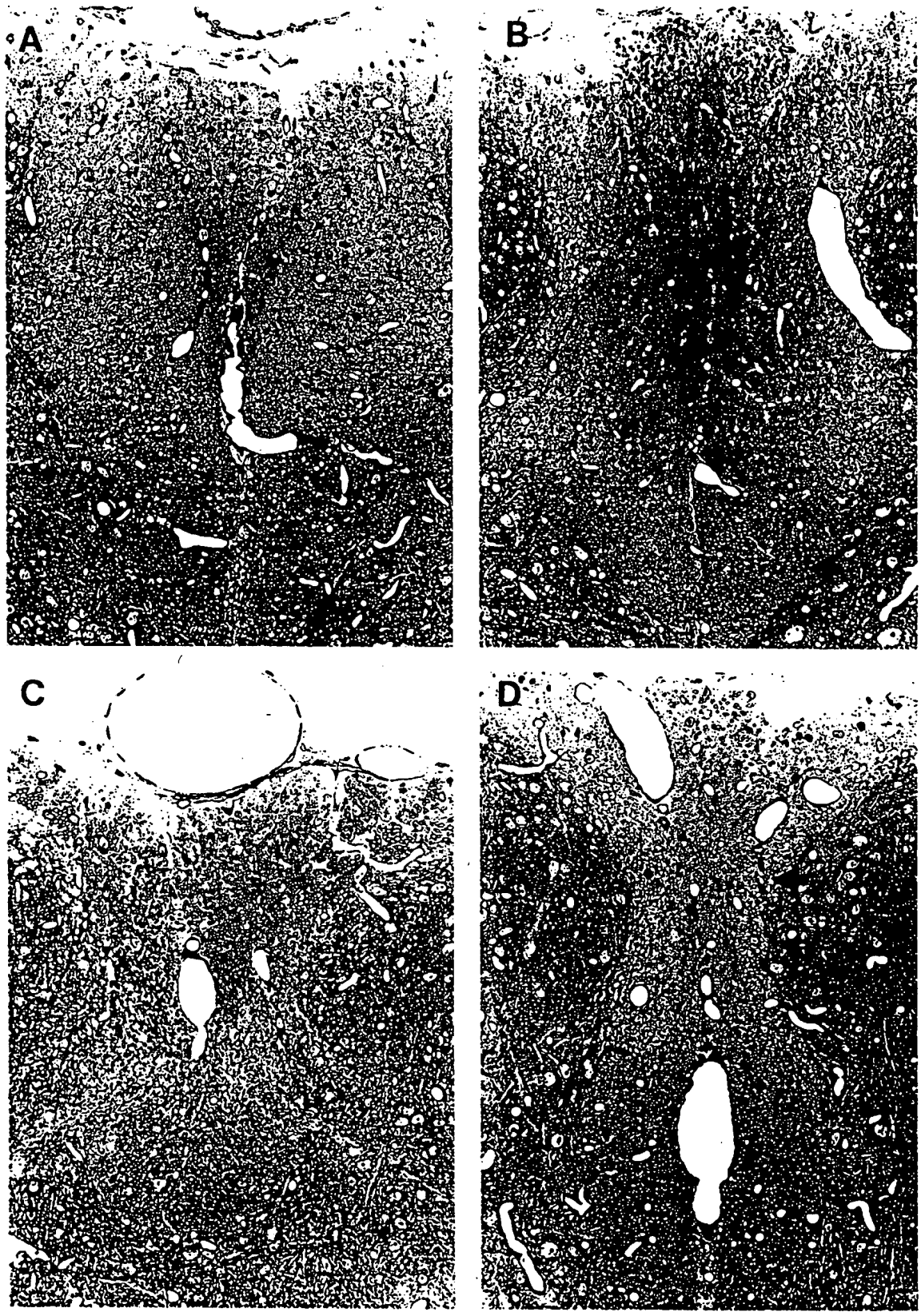


FIG. 1. (A) EGF-responsive neural stem cells in suspension cultures at 14th passage. Numerous large spheres can be seen as well as smaller collections of cells and occasional single cells. Following removal from EGF and dissociation, cells were cultivated for 7 days in 1% serum on poly-*l*-ornithine-coated glass coverslips and then immunolabeled with the antibodies: (B) O4; (C) O1; (D) GalC; (E) MBP; and (F) PLP. Oligodendrocytes are identified by positive labeling with O4, GalC, MBP, and PLP as well as by their morphology. Scale bars, 50 μm.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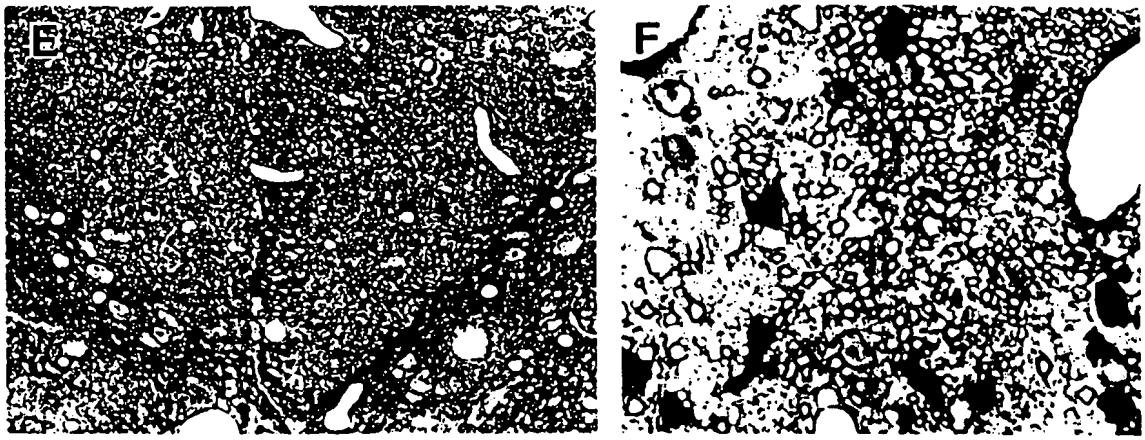


FIG. 2. The dorsal columns (A) of an uninjected *md* rat. No myelinated fibers are present. In (B), EGF-responsive neural stem cells derived from the mouse striatum have myelinated many axons. There is no evidence of rejection of these xenografted cells as a result of the cyclosporin therapy. In (C), an *md* rat injected 17 days earlier with rat striatal neural stem cells also exhibits many myelinated fibers. In (D), EGF-responsive neural stem cells derived from the rat ventral mesencephalon have myelinated scattered axons in the *md* rat CNS. (E, F) Higher magnification of selected areas indicated by arrows in B and D, respectively. (A) Scale bar, 100 μm; (B, C, D) Scale bar, 50 μm.

Stem Cells form Myelin In Vivo

Because neural stem cells are capable of forming oligodendrocyte precursors and oligodendrocytes *in vitro*, we investigated whether these cells would be capable of forming oligodendrocytes which would myelinate CNS axons *in vivo*. Stem cells from different passages were prepared for injection into the *md* rat spinal cords as described under Experimental Methods. Transplantation of cells was only performed on the mutant rats which were readily identified at the time of surgery (6–8 days postnatal) by the pallor of the dorsal columns. The mutant spinal cords appear clear and gelatinous while those of normal littermates were an opaque white. The animals were anesthetized and the spinal cords were exposed at one or two segments and a suspension of the stem cells (either mouse- or rat-derived) in HBSS was injected into the dorsal columns. The animals were allowed to recover from the anesthesia, returned to the dams, and allowed to survive for approximately 2 weeks. Because of the short lifespan of the mutants (approximately 25 days), all of the implanted animals were sacrificed at 21–25 days (approximately 2 weeks postimplant). Upon examination of intact spinal cords, myelination was evident over 3 mm rostral to caudal, in some cases. Histological analysis using toluidine blue-stained transverse sections of the spinal cords 2 weeks after transplantation revealed extensive areas of myelin within the dorsal columns (Figs. 2A–2D). In some of the implanted animals, myelinated fibers were seen throughout the dorsal columns extending as deeply as the margin of the corticospinal tracts (Fig. 2E). Moderate to extensive areas of myelination were seen in 28 of 48 md rats that received cells derived from rat neurospheres and 8 of 12 md rats transplanted with mouse neurospheres. There was no apparent difference in the amount of myelin made by the neurosphere-derived oligodendrocytes from mouse or rat.

When the stem cells are induced to differentiate *in vitro* by removing EGF and adding 1% FBS as described above, the majority of the cells form astrocytes (27). Because of this finding, it was possible that astrogliosis would occur when the stem cells were implanted into the *md* rat spinal cords. To determine whether stem cell-derived astrocytes directly or indirectly caused any astrogliosis, we examined regions of the spinal cords that had received stem cell implants using immunocytochemistry for GFAP. For this analysis, adjacent 1-μm epon sections were stained with toluidine blue to determine the extent of myelination and by immunocytochemistry for GFAP to determine the extent of gliosis. None of the spinal cord sections that contained the injected stem cells exhibited any increase in the number of GFAP immunoreactive cell bodies or processes (i.e., no hyperplasia of astrocytes) when compared to the uninjected *md* rat spinal cords (Figs. 3A–3C). Furthermore, EM analysis of the grafted areas revealed no evidence of hypertrophied astrocyte processes. Together, these data indicate that injection of stem cells into the *md* rat spinal cord results in many of the cells progressing along the oligodendrocyte lineage and does not lead to glial scarring.

Although we have demonstrated a significant amount of myelin formation in the *md* rats, it is possible that their short lifespan limits the extent of myelination that would be possible over a longer period. Because of the short interval between cell injection and sacrifice, it is possible that some precursor cells remain that have the potential to divide and may be capable of myelinating at a later time. Therefore, we performed tritiated

thymidine autoradiography on semi-thin epon sections to determine the extent of cell division in the injected and the uninjected mutants in the regions of myelination. In general, significant numbers of thymidine-labeled cells, that had the light microscopic appearance of oligodendrocytes, were seen only within zones of myelination, with fewer labeled cells within adjacent nonmyelinated regions of the dorsal columns (Fig. 4). This finding suggests that a number of mitotically active donor-derived precursor cells may remain within the recipient *md* spinal cords. It is important to note that we observed no hyperplasia nor hypercellularity as a result of the stem cell injections in any of the animals examined in this study. Although the number of labeled cells seen in the zones of myelination in the injected animals appears to be significantly greater than that seen in uninjected *md* rats, quantitation of labeling indices is necessary for definitive proof that the donor stem cells had retained the ability to divide *in vivo*. However, the increased number of labeled cells within the stem cell implanted *md* CNS suggest that a number of precursor cells may be mitotically active at the time of sacrifice.

Finally, we examined the ultrastructural characteristics of the *md* rat spinal cords that had received stem cell implants (Fig. 5). Numerous mature oligodendrocytes were seen elaborating myelin sheaths around the *md* axons. Such cells are not seen in the uninjected *md* rat and have almost certainly been derived from the transplanted cells. At the ultrastructural level, myelin formed by the donor oligodendrocytes was of normal compaction, possessing both major and minor dense lines, and could be readily distinguished from the small amount of abnormally compacted *md* host myelin (9, 10). There was little evidence of cell death, and no inflammatory cells nor clusters of immature, undifferentiated cells were seen. The ultrastructural analysis revealed no ectopic neurons and no evidence of gliosis as seen in the GFAP staining at the light microscopic level.

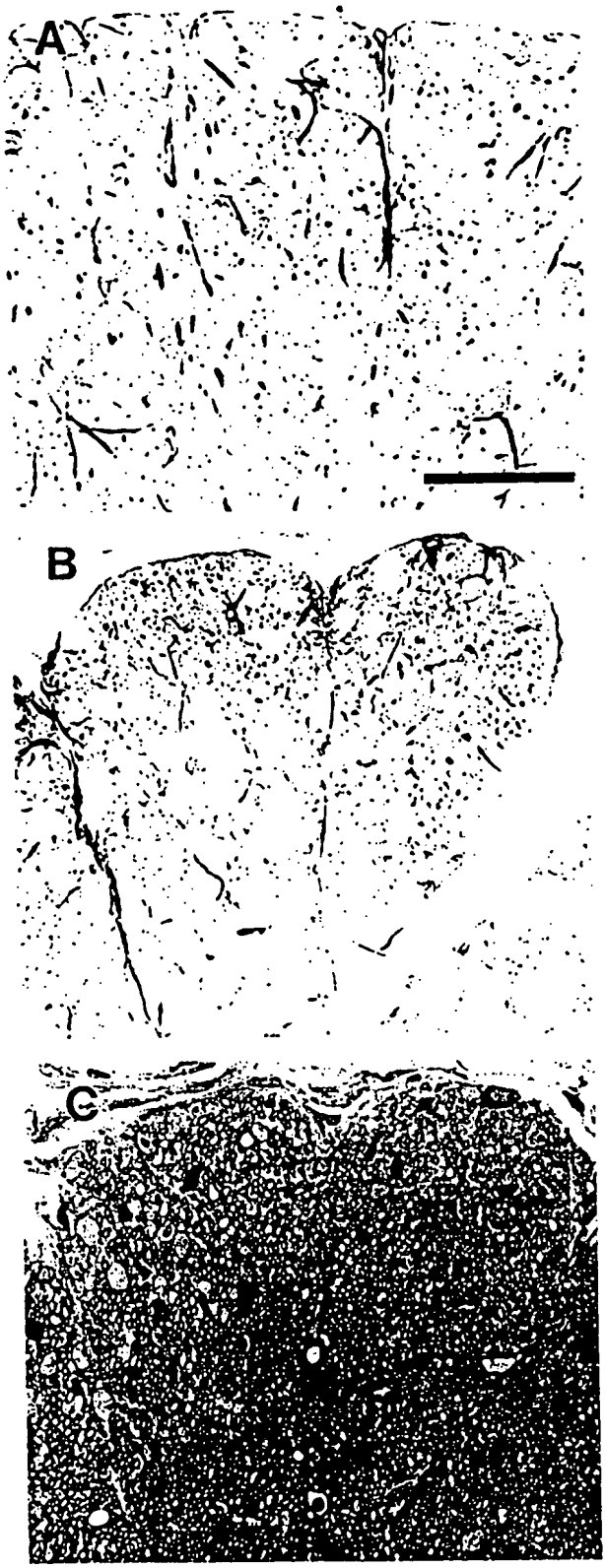
DISCUSSION

We have transplanted EGF-responsive neural stem cells into the spinal cords of *md* rats to determine whether the signals that exist in this glial-deficient environment are capable of influencing oligodendrocyte development from CNS stem cells. Myelin deficiency in these mutant rats is the result of a point mutation (A-C, Thr75-Pro) in the myelin proteolipid protein (PLP) gene and is characterized by a reduction in oligodendrocyte number and a concomitant absence of CNS myelin (8, 12). The absence of normal CNS myelin in this mutant creates an excellent environment in which to examine the myelinating potential of cells with the capacity for oligodendrocyte differentiation. Furthermore, distinction of donor from host cells is considerably more straightforward when compared to other models because of the lack of any repair mechanism from endogenous cells (5, 12). In the *md* model, the small amount of host myelin present is easily identified by the absence of PLP and by an ultrastructural defect in the myelin compaction (9, 10).

Myelination of *md* axons has been achieved by the transplantation of various primary oligodendrocyte and oligodendrocyte precursor preparations as well as immortalized cell lines and Schwann cells (12). These experiments have clearly established that the primary defect is in the oligodendrocyte lineage and that *md* axons are normal in their ability to recruit and support myelinating cells of allogeneic or xenogeneic origin. The multipotential nature of the EGF-responsive stem cells has been clearly demonstrated *in vitro* as they can be induced to differentiate into neurons, astrocytes, and oligodendrocytes after removing EGF from the culture medium (26). For the purposes of these experiments, the stem cell cultures were propagated continuously like a cell line and maintained an undifferentiated phenotype for as many as 25 passages in a fully defined medium containing EGF.

Undifferentiated, nestin-positive stem cells (neurospheres) which lacked expression of differentiated neuronal or glial markers were injected into the dorsal columns of *md* mutant rats at a single thoracolumbar site. The present study shows that when transplanted into the *md* rat, EGF-responsive neural stem cells give rise to mature, myelin-producing oligodendrocytes, capable of ensheathing multiple axons with apparently ultrastructurally normal myelin. This was highly reproducible, both with cells derived from rat and mouse neurospheres. Our failure to detect any ectopic neuron-like cells or to find any evidence of astrocytosis or glial scarring suggests that in this particular myelin-deficient environment, there is a preferential, and appropriately compensatory, differentiation of the multipotential precursor cells along the oligodendrocyte lineage. This suggests that the environment either induces the stem cells to become oligodendrocytes or does not support the other cells that differentiate from the stem cells along an astrocytic or neuronal lineage.

During normal development in the rat CNS, axons in the corticospinal tracts do not become myelinated until approximately 25–30 days postnatal. As noted earlier, all of the *md* animals in these studies were sacrificed prior to 25 days postnatal. In all cases where myelinated fibers were observed deep in the dorsal columns at the margin of the corticospinal tracts, these tracts did not become prematurely myelinated by donor cells. This developmentally appropriate failure to myelinate the corticospinal tracts occurred even though these cells had access to nonmyelinated axons of this late myelinated tract. This strongly suggests not only that



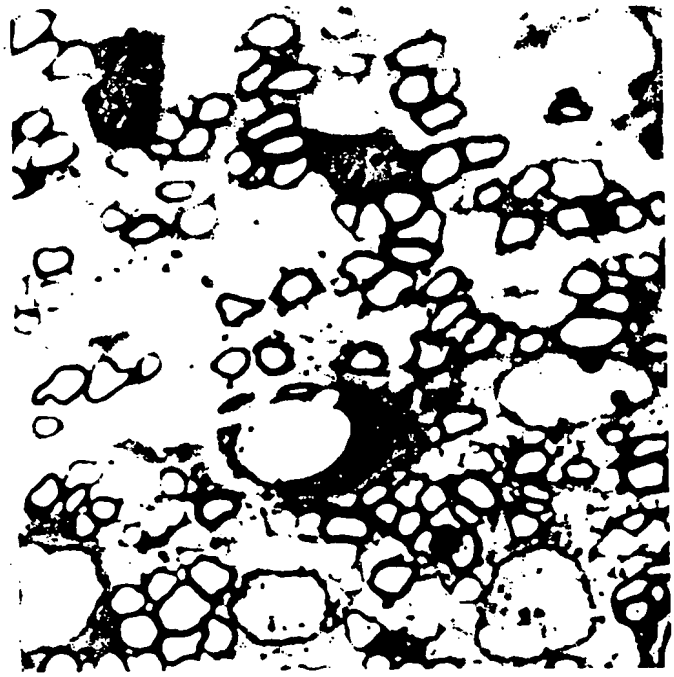


FIG. 4. Light microscopic autoradiograph from a representative area of a transplanted *md* rat spinal cord which demonstrates a large number of myelinated fibers (implanted with rat mesencephalic stem cells). Dividing cells (arrows) have the light microscopic appearance of oligodendrocytes. In the upper left-hand corner, note the mature oligodendrocyte with cytoplasm that appears to be continuous with a number of myelin sheaths. Toluidine blue-stained epon sections. Scale bar, 5 μm.

the mutant environment maintains the ability to regulate oligodendrocyte differentiation in a developmentally and spatially appropriate manner in the face of the oligodendrocyte defect, but furthermore, the differentiated progeny of the implanted stem cells respond appropriately to endogenous cues in the *md* CNS.

Oligodendrocyte differentiation and survival *in vitro* is highly dependent on several trophic factors (3, 4). The yield of oligodendrocytes differentiating from the EGF-responsive neural stem cells *in vitro* can also be influenced by various factors, as CNTF, LIF, and oncostatin M can significantly increase the relative numbers of oligodendrocytes generated (28). Previous evidence suggests that committed oligodendrocyte progenitors may yield more myelin upon transplantation than do mature oligodendrocytes, but it is not

FIG. 3. GFAP (A, B) and toluidine blue (C) stained adjacent sections demonstrate the lack of gliosis in the neural stem cell-implanted *md* rat spinal cords (implanted with rat mesencephalic stem cells). In (A), several millimeters away from the transplanted site, there is little evidence of an increase in GFAP immunoreactivity. Likewise, at the site of transplantation, (B) there is no apparent gliosis. In (C), a serial section from the GFAP-labeled section shown in (B) shows the site of the transplant with numerous myelinated fibers. Scale bar, 20 μm.

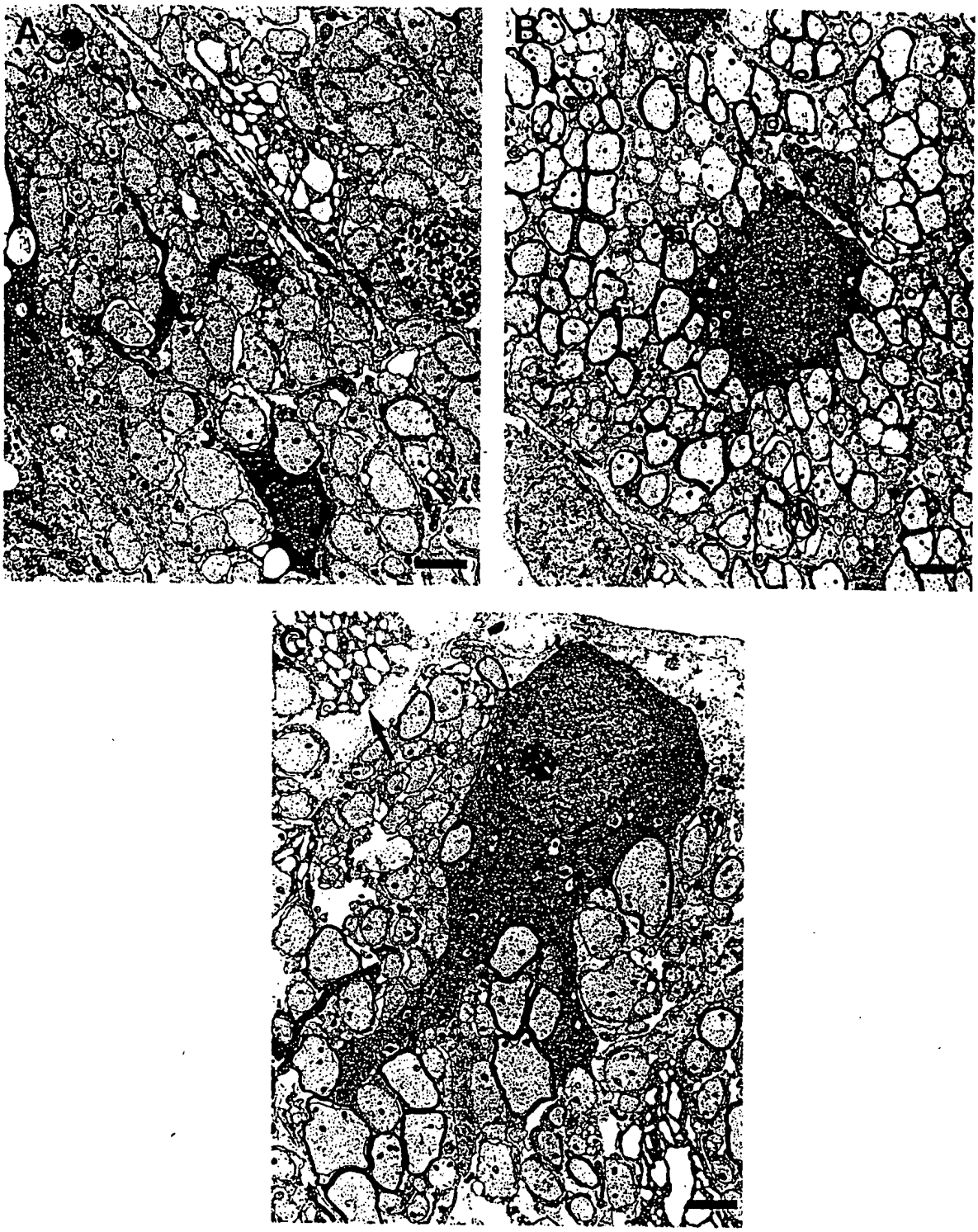


FIG. 5. Electron micrographs from representative myelinated areas in an *md* rat produced by rat neurospheres. (A) Adjacent to the transplant site, most of the axons are nonmyelinated. As in the uninjected mutant, no normal, native oligodendrocytes are present. On the far right in this field, a single degenerating axon is seen. (B) In a transplanted area, the majority of the axons are myelinated with a normal oligodendrocyte present. (C) Underneath the pia, a normal appearing oligodendrocyte has extensive processes leading to many myelinated fibers. Adjacent to this cell, the cytoplasm of an abnormal *md* oligodendrocyte is present (arrow) with distended rough endoplasmic reticulum, a characteristic feature in *md* oligodendrocytes. Scale bars, 2 μm.

known whether different stages in the progression from stem cell to mature oligodendrocyte may offer different selective advantages in achieving optimal myelination. The neural stem cell system provides the unique opportunity to examine the earliest stages of the differentiative progression from a multipotent progenitor to a committed oligodendrocyte precursor (12). Under the standard culture conditions used in the current study, the percentage of cells which differentiate into oligodendrocytes *in vitro* after removal of EGF is small compared to the percentage differentiating into astrocytes or neurons. However, it is interesting to note that this culture system, although strongly favoring neuronal and astrocytic differentiation and not typical of systems normally used in oligodendrocyte culture, nonetheless does support oligodendrocytes for 1 week or longer *in vitro*, albeit in small numbers. Thus, although the extensive analysis involved in screening the many possible growth factor combinations and concentrations to generate an environment optimized for *in vitro* myelin production was beyond the scope of the present study, it is highly likely that appropriate manipulation of *in vitro* conditions prior to transplantion to select for oligodendrocytes and/or their progenitors, could result in a considerable increase in myelin production by the transplanted cells.

The short lifespan of the *md* rat restricted the current studies to a short period following transplantation. Our results suggest that some of the injected stem cells or mitotically active stem cell progeny may survive within the CNS to provide a reservoir potentially capable of generating myelin-forming cells for the remainder of the animal's lifespan; however, proof of this is necessary. In acquired demyelinating disorders, this may provide a population of cells that are able to respond to subsequent demyelination. Even in the short term of the present study, the extent of myelin formation was well beyond that ascribable to passive dispersion (20). This demonstrates that the injected cells or their progeny are able to migrate along the spinal cord. The full migratory potential of these cells remains to be determined as does the stage of differentiation at which migration will occur. Future studies utilizing the longer-lived strain of the *md* rat (13), will allow all the above questions to be addressed more closely, and will permit analysis of whether myelination continues to increase over time and the factors which influence this myelination.

In this report we have extended the observations that the EGF-responsive neural stem cells are multipotential, in that a proportion of the stem cells can differentiate into oligodendrocytes *in vitro* and that these stem cells are capable of differentiating into myelin-forming oligodendrocytes *in vivo* when exposed to the appropriate environment. Behavioral alterations, functional recovery, or a restoration of normal conduction velocity in the injected mutant animals remains to be demonstrated, but a previous study established that conduction velocities can be returned to near normal values by the transplantation of glial progenitor cells (32). This was achieved by myelination similar in extent to that seen in the present study, suggesting that neural stem cells may be equally capable of producing functional improvements.

Transplantation of SV40-immortalized glial progenitors has resulted in sparse myelin formation and the continued proliferation of undifferentiated cells suggesting likely transformation of the injected cells (2). The EGF-responsive neural stem cell would thus appear to be an excellent candidate donor cell for use in human therapies for myelin disease. Large numbers of homogeneous cells can be generated as required, minimizing ethical considerations. Lines can be screened for the presence of known adventitious agents. Finally, the cells are stimulated to divide using growth factors rather than oncogenes. This is a substantial advantage since little is known about the safety and long-term stability of cell lines immortalized with various oncogenes, either conditionally immortalized, as in the case of temperature-sensitive oncogenes, or driven by other inducible promoter systems. In addition to the obvious and most disturbing risks of malignancy, overproliferation may disrupt normal cytoarchitecture in areas adjacent to the transplant site and there is evidence that such cell lines may over time exhibit diminished myelinating capacity. One study has reported such a diminution and in addition, compression of normal adjacent tissue within 1 month of transplantation of growth factor-expanded glial cells into rat spinal cord (16). In contrast, we observed no hyperplasia as a result of the stem cell injections in any of the animals in this study. While the period between stem cell injection and sacrifice was relatively short, no abnormalities in the growth or proliferation of the stem cells was seen in any of the injected mutants. Light and electron microscopic analyses indicated that the stem cells were highly efficient in forming myelin. There was no evidence of large numbers of undifferentiated stem/precursor cells remaining in areas of hypomyelination as seen in some previous studies (16). Extensive myelination, up to 3 mm rostral or caudal from the single site of stem cell injection, was achieved even after 25 passages *in vitro*, offering the prospect of considerable expansion of a source population without loss of myelinating capacity. This would be of particular advantage when the available source population is limited, as is the case for human cells.

The possibility of xenografting should not be discounted when considering the use of transplantation as a treatment for human neurodegenerative diseases, particularly in view of the continuing advances in immunosuppressive regimes. Allografting, however, re-

mains the approach of choice. EGF-responsive neural stem cells with similar *in vitro* characteristics have recently been isolated from fetal human brain (25, 6, 30). If these human stem cells also possess the capacity to divide and differentiate into oligodendrocytes *in vivo*, they may prove useful in replacement therapies for human dysmyelinating and demyelinating diseases, possibly in conjunction with appropriate immunosuppressive therapy.

In conclusion, our results suggest that EGF-responsive neural stem cells are capable of safe, extensive myelination *in vivo* in a manner appropriately responsive to relevant signaling by the recipient environment. Their potential for *in vivo* expansion from a surviving reservoir of transplanted stem or progenitor cells make them a leading candidate to be considered for eventual use in therapeutic transplantation into human patients.

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Incorporation and Glial Differentiation of Mouse EGF-Responsive Neural Progenitor Cells after Transplantation into the Embryonic Rat Brain

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***In vitro*, epidermal growth factor (EGF)-responsive neural progenitor cells exhibit multipotent properties and can differentiate into both neurons and glia. Using an *in utero* xenotransplantation approach we examined the developmental potential of EGF-responsive cells derived from E14 mouse ganglionic eminences, cortical primordium, and ventral mesencephalon, after injection into the E15 rat forebrain ventricle. Cell cultures were established from control mice or from mice carrying the lacZ transgene under control of the promoters for nestin, glial fibrillary acidic protein (GFAP), or myelin basic protein (MBP). The grafted cells, visualized with mouse-specific markers or staining for the reporter gene product, displayed widespread incorporation into distinct forebrain and midbrain structures and differentiated predominantly into glial cells. The patterns of incorporation of cells from all three regions were very similar without preference for the homotopic brain areas. These results suggest that EGF-responsive progenitor cells can respond to host derived environmental cues, differentiate into cells with glial-like features, and become integrated in the developing recipient brain.**

***Key Words:* stem cells; progenitor cells; EGF; *in utero* grafting; differentiation; glia; transgenic mice.**

INTRODUCTION

The generation of cellular diversity during development of the mammalian brain has become a subject of major interest during recent years. Early in development, precursor cells can be isolated from the neural crest that are capable of differentiation into both neurons and glia (Stemple and Anderson, 1992). However, later in ontogeny, the lineage potential of precursor cells, the generation of cellular diversity, and the role of multipotent precursor cells in brain development are more controversial. In cell culture studies, several investigators have reported the presence of multipotent precursor cells in both mid- and late-gestation embryonic CNS (Reynolds *et al.*, 1992; Kilpatrick and Bartlett, 1993; Davies and Temple, 1994; Williams and Price, 1995), as well as in the brain of adult rodents (Reynolds and Weiss, 1992; Richards *et al.*, 1992; Gage *et al.*, 1995a; Palmer *et al.*, 1995; Gritti *et al.*, 1996, Palmer *et al.*, 1997; for review see Gage *et al.*, 1995b; Kilpatrick *et al.*, 1995; Weiss *et al.*, 1996). On the other hand, *in vivo* studies using retroviral vectors carrying marker genes such as β-galactosidase have shown that in the embryonic brain most progenitor cells are restricted to a specific fate (neuronal or glial) while the percentage of pluripotent cells is very low (Luskin *et al.*, 1988; Price and Thurlow, 1988; Grove *et al.*, 1993; Luskin *et al.*, 1993; Walsh and Cepko, 1992, 1993; Reid *et al.*, 1995).

Recently, several groups have shown that *in utero* transplantation of cell suspensions from primary tissue might allow for the determination of the developmental capacity of progenitor cells (Brüstle *et al.*, 1995; Campbell *et al.*, 1995; Fishell, 1995). Diverging results of these studies and difficulties of interpretation might be due to the fact that the cell suspensions contained a mixture of cells in different states of commitment and differentiation and thus suggest that isolation of progenitor cells from the embryonic brain might be necessary in order to distinguish the role of multipotent progenitors from that of lineage-restricted or postmitotic cells.

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Neural progenitor cells can be isolated from the mammalian brain and induced to proliferate *in vitro* in the presence of epidermal growth factor (EGF; Anchan *et al.*, 1991; Reynolds *et al.*, 1992; Reynolds and Weiss, 1992, 1996; Vescovi *et al.*, 1993; Svendsen *et al.*, 1995). The EGF-stimulated cells can be grown into spheres ("neurospheres") which are positive for the progenitor marker nestin but not for standard glial or neuronal markers (Von Visger *et al.*, 1994; Reynolds and Weiss, 1996; Schinstine and Iacovitti, 1996; Carpenter *et al.*, 1997). By long-term clonal analysis these EGF-responsive neurosphere cells have been shown to exhibit the common features of stem cells, i.e., long-term proliferation and self-renewal, long-term multipotency, and the production of a large number of progeny (Reynolds and Weiss, 1996).

The purpose of the present study was to investigate the *in vivo* properties of *in vitro* propagated EGF-dependent progenitor cells after reintroduction of the cells into the embryonic brain environment. Using an *in utero* xenotransplantation approach, we injected EGF-responsive neural progenitor cells, derived from different areas of the embryonic E14 mouse brain, into the E15 rat forebrain ventricle. Independent of origin, the cells exhibited widespread incorporation into the brain parenchyma which suggests that these cells along the rostrocaudal axis of the subventricular zone are not regionally or positionally specified. There was clear evidence of donor cell differentiation into astrocytes, but not into neurons, which suggests that the developmental potential of these cells is more restricted *in vivo* when compared to previous *in vitro* results.

RESULTS

Characterization of EGF-Responsive Cells in Vitro

EGF-responsive neural progenitor cells were isolated from the ganglionic eminences, the cortical primordium, and the ventral mesencephalon of nontransgenic E14 mice and expanded in cell culture for 5 weeks, during which time they were passaged five times. Additional cultures were obtained from the ganglionic eminences of transgenic mice carrying the lacZ gene under control of the promoters for nestin (Zimmermann *et al.*, 1994), glial fibrillary acidic protein (GFAP; Brenner *et al.*, 1994), or myelin basic protein (MBP; Wrabetz *et al.*, 1998).

In vitro, the cells divided every 2 to 3 days as observed previously (Reynolds and Weiss, 1996; Carpenter *et al.*, 1997; Svendsen *et al.*, 1997). Cells derived from different brain regions grew similarly and no differences were observed in the growth rate of the transgenic vs the nontransgenic cells. The cells used for transplantation were positive for the neural progenitor marker nestin but did not stain for glial or neuronal markers such as GFAP, M2, 04, PLP, or β-tubulin, which is in accordance with previous observations (Von Visger *et al.*, 1994; Reynolds and Weiss, 1996; Schinstine and Iacovitti, 1996; Carpenter *et al.*, 1997).

To determine the capacity of the cells to differentiate into the major CNS phenotypes, the EGF-responsive cells were plated on polyornithine-coated slides and exposed to 1% fetal bovine serum in the absence of EGF. Cultures propagated for 5 weeks *in vitro* before differentiation exhibited staining for neurons, oligodendrocytes, and astrocytes (Fig. 1), indicating sustained multipotentiality of the cells used for transplantation. In cultures grown for up to 10 weeks before differentiation, 5–7% of the cells consistently stained for the neuronal marker β-tubulin, while there was a qualitative decrease in the number of neurons after this time point (Carpenter, unpublished observations). *In vitro*, all the astrocytes, as seen by GFAP-immunoreactivity, were simultaneously stained for the mouse glial marker M2 (Figs. 1A and 1B). In astrocytes derived from the GFAP-lacZ transgenic mice, the transgene and the GFAP protein were expressed simultaneously, as were the MBP-lacZ transgene and the MBP protein in oligodendrocytes derived from the MBP-lacZ transgenic mice (Figs. 1E and 1F). This is in accordance with previous studies which had shown that the lacZ transgene under control of the nestin, GFAP, and MBP promoters was appropriately regulated in a cell-specific fashion (Brenner *et al.*, 1994; Hammang *et al.*, 1994; Zimmermann *et al.*, 1994; Wrabetz *et al.*, 1998).

Widespread Incorporation of Grafted Mouse Cells

After repeated passages *in vitro* the progenitor cells were transplanted into the forebrain ventricle of E15 rats. Five weeks posttransplantation the distribution and differentiation of the transplanted cells were analyzed using the mouse-specific antibodies M2 and M6, β-galactosidase immunohistochemistry (to detect the reporter gene product), and *in situ* hybridization for mouse satellite DNA. In 59 animals, the transplanted cells had incorporated extensively into the host brain parenchyma. The majority of the cells were located in grey matter with a few cells evident in white matter tracts.

Progenitor cells derived from the ganglionic eminences were found widespread in telencephalic, diencephalic, and mesencephalic structures (Figs. 2–6, Table 1). Interestingly, the cells did not incorporate homotopi-

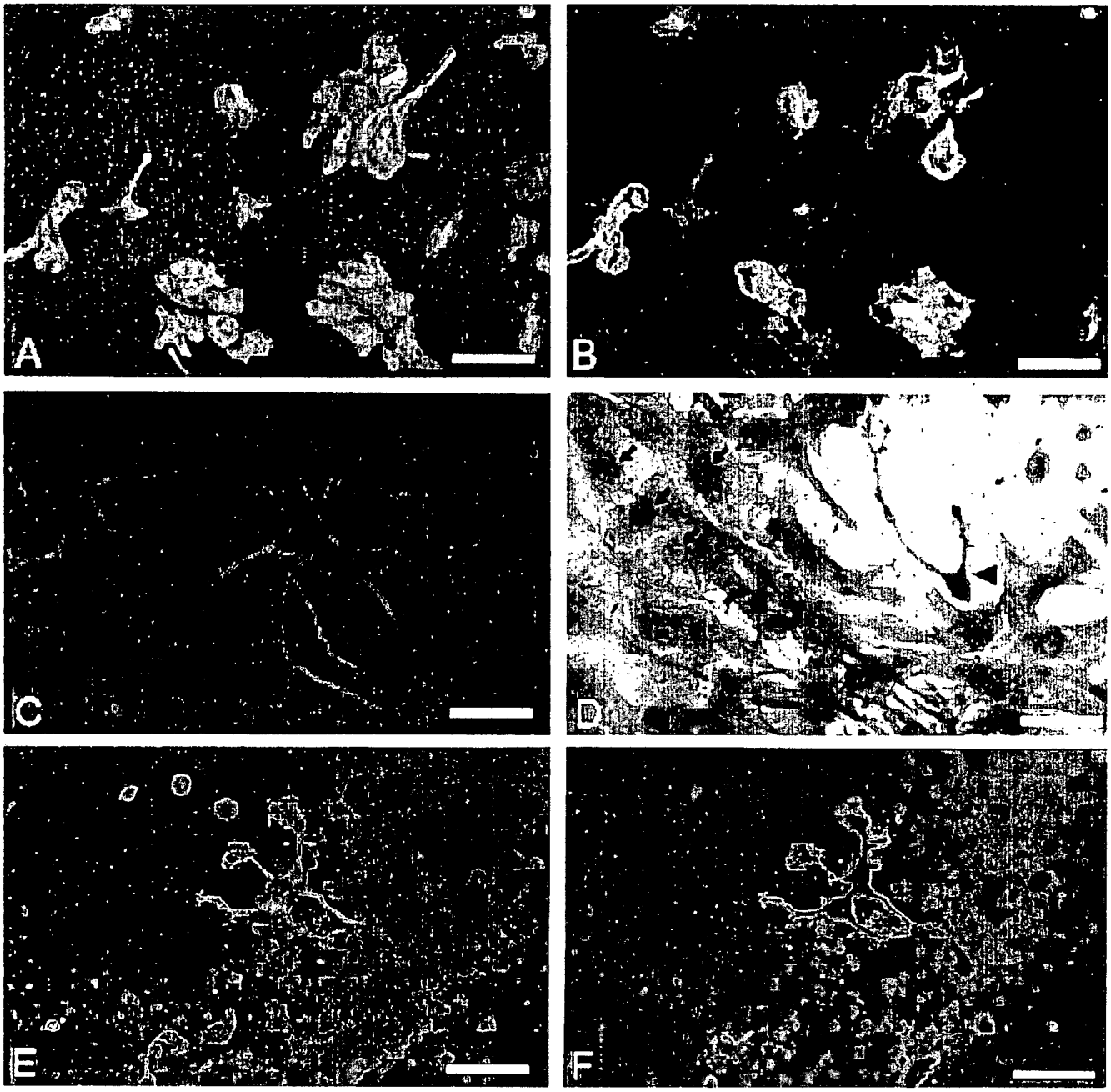


FIG. 1. Immunocytochemical characterization of EGF-responsive neural progenitor cells. Differentiation of the EGF-responsive cells into astrocytes (A, B, D), neurons (C), and oligodendrocytes (D–F) *in vitro*. (A) All astrocytes expressing GFAP (A) simultaneously label for the mouse glial marker M2 (B). (C) β-Tubulin staining several neurons. (D) Triple labeling for GFAP (brown–red), PLP (black), and the GFAP-lacZ reporter gene product (bluish) in cells derived from GFAP-lacZ transgenic mice. Astrocytes show appropriate expression of the transgene, which is located in the nucleus (arrows), while the PLP-positive oligodendrocyte (arrowhead) does not show transgene expression. (E) Oligodendrocytes derived from MBP-lacZ transgenic mice simultaneously stain for the MBP-lacZ reporter gene product (E) and for MBP (F). Scale bars: A–C and E and F, 100 μm; D, 50 μm.

TABLE 1
Distribution of EGF-Responsive Neural Progenitor Cells Derived from the Mouse Ganglionic Eminences after Transplantation into the Developing Rat Brain

Animal no.	Olfactory bulb	Cortex	Striatum	Septum	Hippocampus	Thalamus	Hypothalamus	Midbrain
1	ND	−	−	−	−	+	+	−
2	ND	−	−	−	−	+	+	+
3	ND	−	−	−	−	++	−	+++
4	ND	−	−	−	+	−	−	++
5	++	+	−	+	+++	+	++	+
6	ND	−	−	−	−	+++	+	++
7	ND	+	−	−	+	+	++	++
8	ND	+++	−	−	++	−	−	+
9	ND	−	−	−	+	+	+	++
10	ND	−	+	−	+	−	+++	++
11	ND	−	−	−	+++	−	−	−
12	ND	−	−	−	+	++	+	+++
13	ND	+	−	−	+++	+	+	++
14	−	−	−	−	+	−	+	++
15	+	++	++	−	+	+	++	++
16	++	+	+	+	−	++	++	+++
17	−	−	−	−	++	++	−	−
18	+	++	−	−	+	+	+	+
19	+	+	−	−	+	+	−	++
20	−	−	−	−	−	+	+	++
21	−	+	+	−	−	+	+	+
22	−	−	−	−	+	−	+	−
23	−	+	−	−	−	+	+	+++
24	+	+	−	+	+	+	++	+
25	++	+	−	+	+	−	++	++
26	++	−	+	−	−	+	+	++
27	−	++	−	−	−	−	++	+
28	−	+	−	−	+	−	+	++
29	−	+	−	−	+	−	−	−
Transplant	8/17	15/29	5/29	4/29	19/29	19/29	22/29	24/29
Median	−	+	−	−	+	+	+	++

Note. Incorporation of EGF-responsive progenitors derived from the mouse E14 ganglionic eminences after injection into the E15 rat forebrain ventricle. The analysis was performed on sagittal sections processed for immunohistochemistry with the M2 or M6 antibodies; *in situ* hybridization for mouse satellite DNA was performed on selected sections to confirm the results obtained by immunohistochemistry. The animal numbers 1–11 correspond to rat pups receiving transplants of cells derived from nontransgenic mice. Animals 12–17 received cells from transgenic mice carrying the lacZ gene under control of the nestin promoter, animals 18–22 received cells from GFAP-lacZ transgenic mice, and animals 23–29 cells from MBP-lacZ transgenic cells. +++, rich incorporation (dense M2 staining that covered at least one-fourth of the cross sectional area of the structure as shown in Fig. 1 for the inferior colliculus); ++, moderate incorporation (many clusters of M2-positive cells that covered at least one-tenth of the cross sectional area of the structure described); +, scattered clusters of transplanted cells; −, no detectable staining; ND, not determined.

cally into the striatum or the globus pallidus and only rare clusters of cells were observed in these structures. Brain regions which exhibited the most significant extent of incorporation for both nontransgenic and transgenic cells were hippocampus (Figs. 5A–5C), thalamus, hypothalamus (Figs. 4, 5D, and 5E), and mesencephalon, primarily the inferior colliculus (Figs. 2 and 3, Table 1). To a lesser extent, cells were observed in the cerebral cortex and in the olfactory bulb (Fig. 6). In some regions integration was frequently confined to specific subareas, e.g., the mamillary body of the hypothalamus or the external plexiform layer of the olfactory bulb. In the thalamus, the cells had aggregated in subnuclei of the structure, most of which were localized close to the third ventricle. By contrast, no preference for specific layers could be observed in the cortex and hippocampus. In some cases, the likely migratory path of the cells could be deduced, e.g., along the forceps minor of the corpus callosum into the frontal cortex and along the anterior commissure toward the olfactory bulb (Fig. 6C).

Progenitor cells derived from the cortical primordium exhibited a pattern of incorporation that was very similar to that of the ganglionic eminence-derived cells (Table 2). Cortical cells showed slightly more extensive incorporation into the cortex and fewer cells were observed in the thalamus, but otherwise no differences

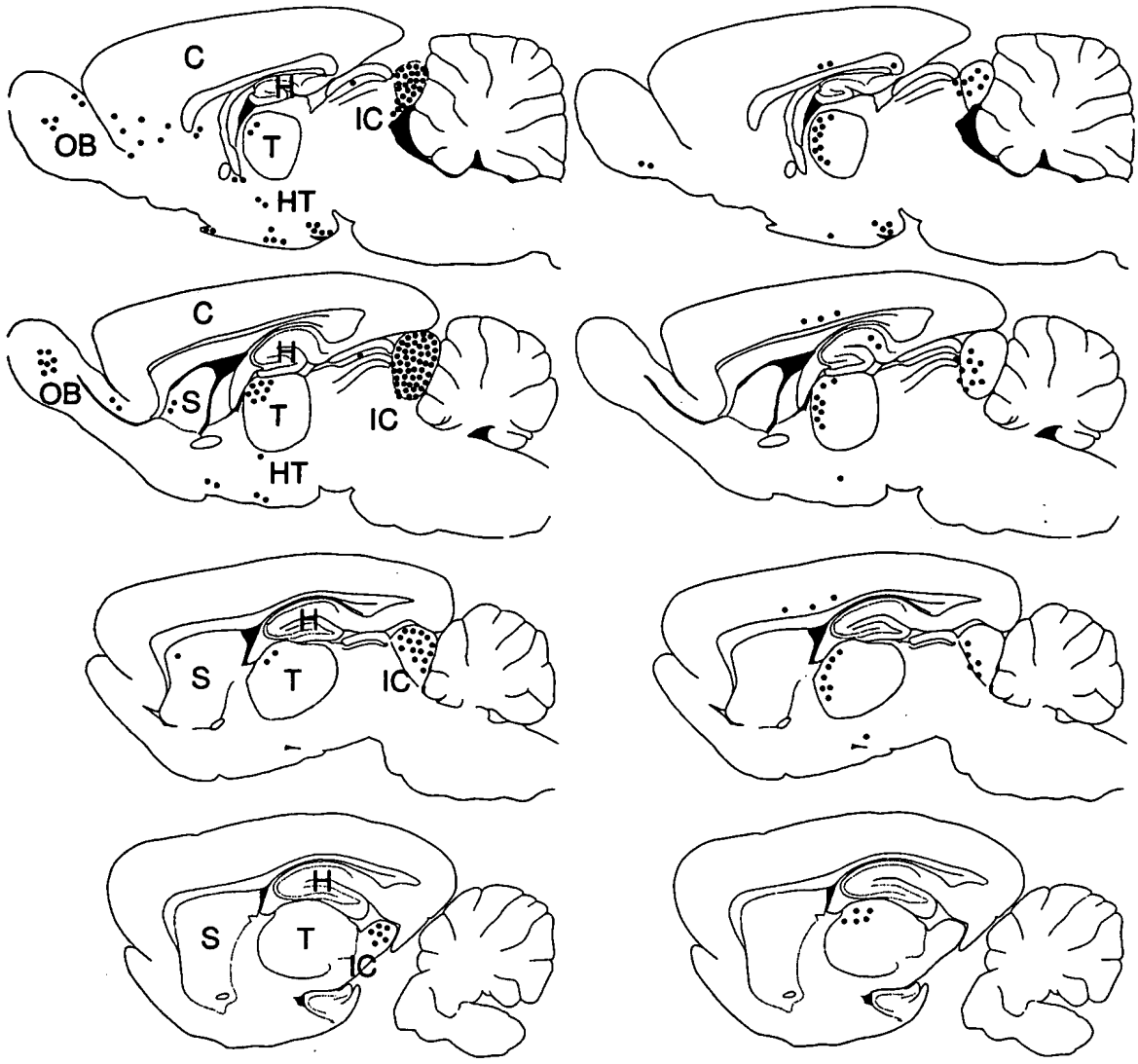


FIG. 2. Distribution of transplanted cells. EGF-responsive neural progenitor cells in two representative recipient brains, as seen by immunohistochemistry with either M2 or M6 and by *in situ* hybridization for mouse satellite DNA. The sagittal sections on the left correspond to animal No. 16 in Table 1, which received a transplant of progenitor cells from the ganglionic eminences; the sections on the right correspond to animal No. 11 in Table 2, which received cells from the cortical primordium. The dots represent clusters of cells rather than single cells (and thus not absolute cell numbers) and the relative proportions of mouse cells incorporated into the different brain areas. (C, cortex; H, hippocampus; HT, hypothalamus; IC, inferior colliculus; OB, olfactory bulb; S, striatum; T, thalamus.)

could be observed (Table 2). Similarly, progenitor cells derived from the ventral mesencephalon did not show any preference for mesencephalic structures but were able to incorporate into telencephalic structures similar to the cells derived from the telencephalon. The overall number of incorporated cells was lower but the overall pattern of incorporation was the same (Table 3). Thus, progenitor cells derived from different parts of the rostrocaudal axis of the embryonic brain, after transplantation into the forebrain ventricle of age-matched recipients, showed similar widespread patterns of incorporation without obvious preference for their homotopic area.

Differentiation into Glial Cells

Transplanted cells were exclusively found in the parenchyma; cells were found neither in the ventricle nor attached to the ventricular walls. Immunohistochemistry for the nestin-lacZ transgene did not show any labeled cells. Furthermore, in areas with dense M2 staining, cells did not stain for the progenitor markers

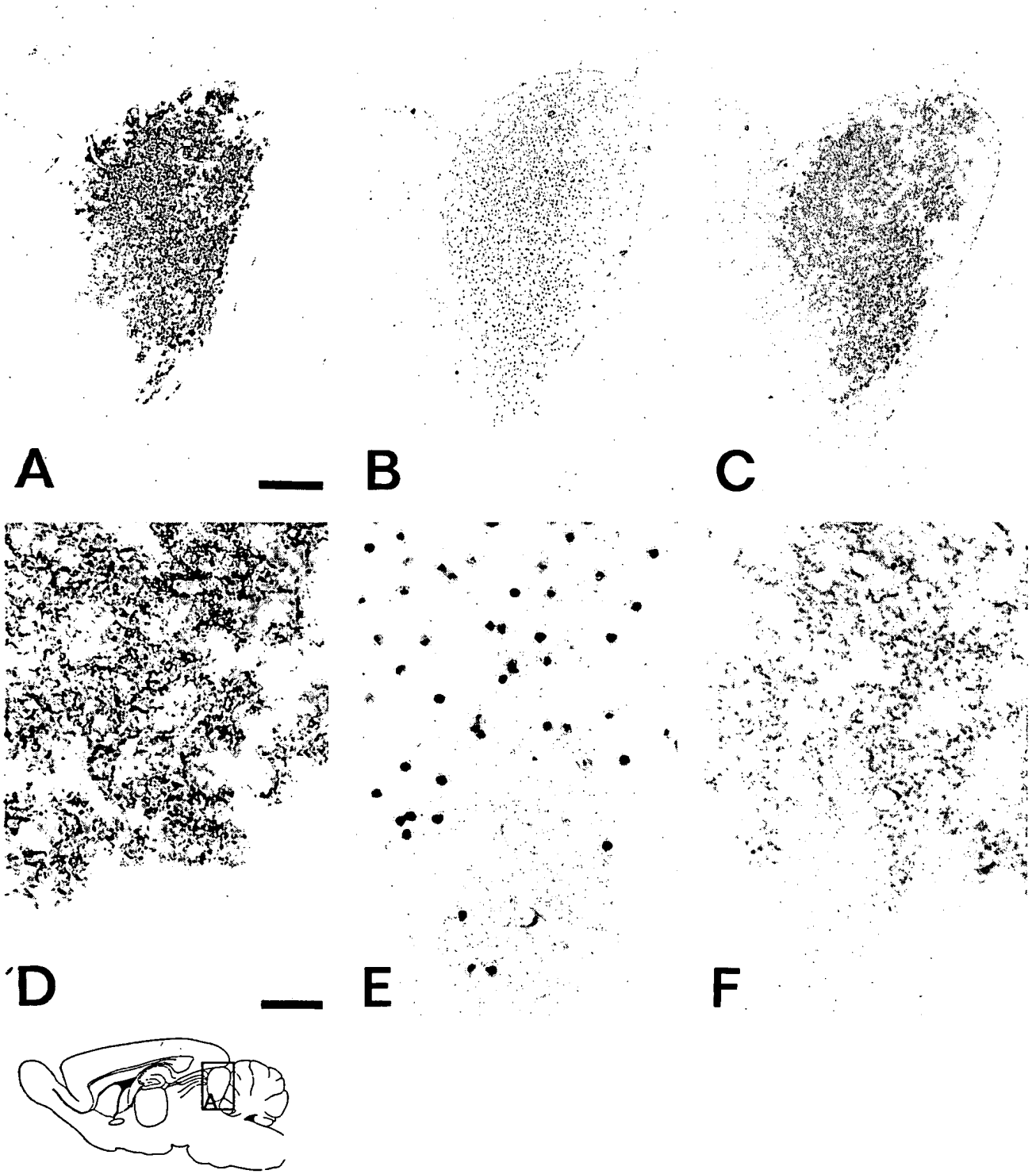


FIG. 3. Overlapping patterns of M2- and M6-positive cellular profiles and *in situ* hybridization for mouse satellite DNA. (A) M2-positive cellular profiles in a sagittal section of the inferior colliculus. The whole structure is covered by the transplanted cells. (B) Mouse nuclei stained by *in situ* hybridization for mouse satellite DNA in a section adjacent to A. (C) M6-positive cellular profiles in a section adjacent to A and B. (D) High-power view of A. (E) High-power view of B showing mouse nuclei of homogenous size. (F) High-power view of C. Scale bars: A, 500 μm; D, 60 μm.

TABLE 2

Distribution of EGF-Responsive Neural Progenitor Cells Derived from the Mouse Cortical Primordium after Transplantation into the Developing Rat Brain

Animal no.	Olfactory bulb	Cortex	Striatum	Septum	Hippocampus	Thalamus	Hypothalamus	Midbrain
1	+++	++	+	−	−	−	+	++
2	−	−	−	−	−	−	+	+++
3	−	+	+	−	+	+	−	−
4	−	−	−	−	−	+++	−	−
5	−	−	−	−	−	+	+	++
6	++	+	+	−	++	−	−	+
7	+	−	−	−	−	−	+	++
8	−	++	−	−	+++	−	−	+
9	−	++	−	+	−	−	++	++
10	++	−	−	−	++	+	−	+
11	+	++	−	+	+	+++	++	++
12	−	−	−	−	+	−	+	+
13	−	+	−	−	+	+	+	++
14	++	+	−	−	++	−	+	+++
Transplant	6/14	8/14	3/14	2/14	8/14	6/14	9/14	12/14
Median	−	+	−	−	+	−	+	++

Note. Incorporation of EGF-responsive progenitors derived from the mouse E14 cortical primordium after injection into the E15 rat forebrain ventricle. For details, see legend to Table 1.

nestin or vimentin, thus suggesting that the transplanted cells did not remain in an immature state. Immunohistochemistry for the mouse-specific glial cell marker M2 revealed differentiation into astrocytes. In accordance with previous observations, the M2 antibody labeled the membrane of the mouse cells leaving the cytoplasm unstained, which facilitates the identification of cell types by their processes and the size of the unstained cell bodies (Lund *et al.*, 1989; Wictorin *et al.*, 1991; for extensive discussion about this antibody see

TABLE 3

Distribution of EGF-Responsive Neural Progenitor Cells Derived from the Mouse Ventral Mesencephalon after Transplantation into the Developing Rat Brain

Animal no.	Olfactory bulb	Cortex	Striatum	Septum	Hippocampus	Thalamus	Hypothalamus	Midbrain
1	++	++	−	−	+	−	+	++
2	−	+	−	−	+	−	+	+
3	++	−	−	−	++	−	+	++
4	−	+	−	−	++	−	−	−
5	−	−	−	−	+	+	+	+
6	−	+++	−	−	+++	−	+	++
7	++	+++	−	−	+++	−	+	+
8	−	+	−	−	+	−	−	−
9	+	−	−	−	−	−	+	−
10	−	−	+	−	−	−	++	++
11	−	++	−	−	−	−	++	++
12	+	+	−	−	−	−	−	+
13	−	−	−	+	−	+	−	++
14	−	−	−	−	−	−	−	+
15	−	+	−	−	+	−	−	++
16	−	−	+	−	−	−	+	+
17	−	+	−	−	−	−	−	+
18	++	−	−	−	−	−	−	−
Transplant	6/18	10/18	2/18	1/18	9/18	2/18	12/18	14/18
Median	−	+	−	−	+/−	−	+	+

Note. Incorporation of EGF-responsive progenitors derived from the mouse E14 ventral mesencephalon after injection into the E15 rat forebrain ventricle. For details, see legend to Table 1.

Campbell *et al.*, 1995, or Gates *et al.*, 1998). In grey matter, the M2-positive profiles had small round to slightly oval-shaped cell bodies (10–12 μm) and short, frequently ramified processes radiating in all directions (Figs. 3D, 4B, 4D, and 6B), similar to what is seen in protoplasmic grey matter astrocytes in Golgi–Hortega staining (for review see Privat *et al.*, 1995). In most areas, the processes showed increased M2 immunoreactivity and variability in caliber, while in cortex and hippocampus some cells showed thin delicate processes (Fig. 6B).

In contrast to the *in vitro* results, M2-positive cells did not show colocalization with either GFAP or S-100β *in vivo*. In addition, immunohistochemistry for the GFAP-lacZ transgene did not show any labeled cells. *In vivo*, only a subset of the total astrocyte population shows labeling with GFAP and/or S100β (Bignami and Dahl, 1976; Ludwin *et al.*, 1976), and these markers are considered to stain reactive and presumably more immature astrocytes (Isacson *et al.*, 1987; Miyake *et al.*, 1989; Lundberg *et al.*, 1996). In accordance with this, after transplantation of EGF-responsive progenitor cells derived from GFAP-lacZ transgenic mice into the adult rat striatum (which causes a transplant-induced upregulation of GFAP), the transplanted cells coexpressed the GFAP-transgene and M2 (Winkler *et al.*, manuscript in preparation). As shown by Zhou *et al.* (1990), such cells coexpressing GFAP and M2 had the typical appearance of reactive astrocytes with small nuclei and few, thick processes rather than the extensive highly ramified arborizations we observed in our *in utero* transplant paradigm. Thus, the lack of expression of GFAP in the M2-positive cells might reflect a nonreactive or more mature state of the glial cells studied here.

In white matter tracts (e.g., in the corpus callosum and in the hippocampal region) some M2-positive profiles showed a slightly different morphology with oval cell bodies and elongated thin processes which oriented parallel to the white matter tracts (Figs. 5A, 5B, 6C, and 6D) These cells were reminiscent of oligodendrocytes (Butt and Ransom, 1989; Szuchet, 1995); however, in animals that had received cells from MBP-lacZ transgenic mice, immunohistochemistry for the reporter gene product did not label any cells. In contrast, oligodendrocytes derived from the MBP-lacZ transgenic cells after transplantation into the neonatal and the adult brain (Winkler and Fricker, manuscript in preparation) stained for the MBP-lacZ transgene, thus suggesting that the M2-positive profiles observed in this experiment are likely protoplasmatic astrocytes which have integrated into the white matter (for review see Raisman *et al.*, 1985; Privat *et al.*, 1995).

No Obvious Differentiation into Neuronal Cells

In contrast to the *in vitro* results, there was no evidence of neuronal differentiation of the transplanted progenitor cells. The M6 antibody is known to stain neurons and their processes (Lund *et al.*, 1985, 1993; Zhou *et al.*, 1990; Campbell *et al.*, 1995; Olsson *et al.*, 1997), but in cell culture oligodendrocytes and astrocytes have also been observed to be partially stained by M6 (Lagenauer *et al.*, 1992; for extensive discussion about this antibody see Campbell *et al.*, 1995, or Olsson *et al.*, 1997). Thus, staining of subsets of glial cells cannot be excluded. In all animals the distributions of M6- and M2-stained cells were very similar and showed clear overlap (Figs. 3A, 3C, 4C, and 4E). Most M6-positive profiles, as with M2 staining, were glial-like with short ramifying processes, however, with no processes seen to extend from the regions of M6-positive cells (Figs. 3F and 4E). Furthermore, the size of the M6-positive cellular profiles was small (10–12 μm) and not in a range of about 15–20 μm as reported previously for neurons stained with the M6 antibody (Campbell *et al.*, 1995; Olsson *et al.*, 1997).

The entire population of grafted mouse cells, independent of their degree of differentiation or their expression of mouse-specific markers, was detected by *in situ* hybridization for mouse satellite DNA (Brüstle *et al.*, 1995). Labeled cells were exclusively detected in areas stained for M2 and M6 and all subnuclei showed an identical overlapping pattern in M2 or M6 staining and *in situ* hybridization (Figs. 3, 5D, 5E, 6F, and 6G), indicating that the grafted mouse progenitor cells did not integrate into any area other than those already detected by the M2 and M6 antibodies. The stained nuclei gave the impression of a homogenous glial-like cell population with small round nuclei (Figs. 3E, 5E, and 6G).

DISCUSSION

The EGF-responsive neural progenitor cells used for *in utero* transplantation in the present study were isolated from three different regions, i.e., cortex, striatum, and ventral mesencephalon of the E14 embryonic mouse brain. Reynolds and Weiss (1992, 1996) have shown that repeated passages during extended cell culture in the presence of EGF, as used here, serve to enrich a proliferative cell population free of differentiated cells. The present results show that EGF-responsive progenitors injected into the forebrain ventricle of rat embryos are able to pass across the ventricular wall, become incorporated into widespread areas of the telencephalon, diencephalon, and mesencephalon, and differentiate into

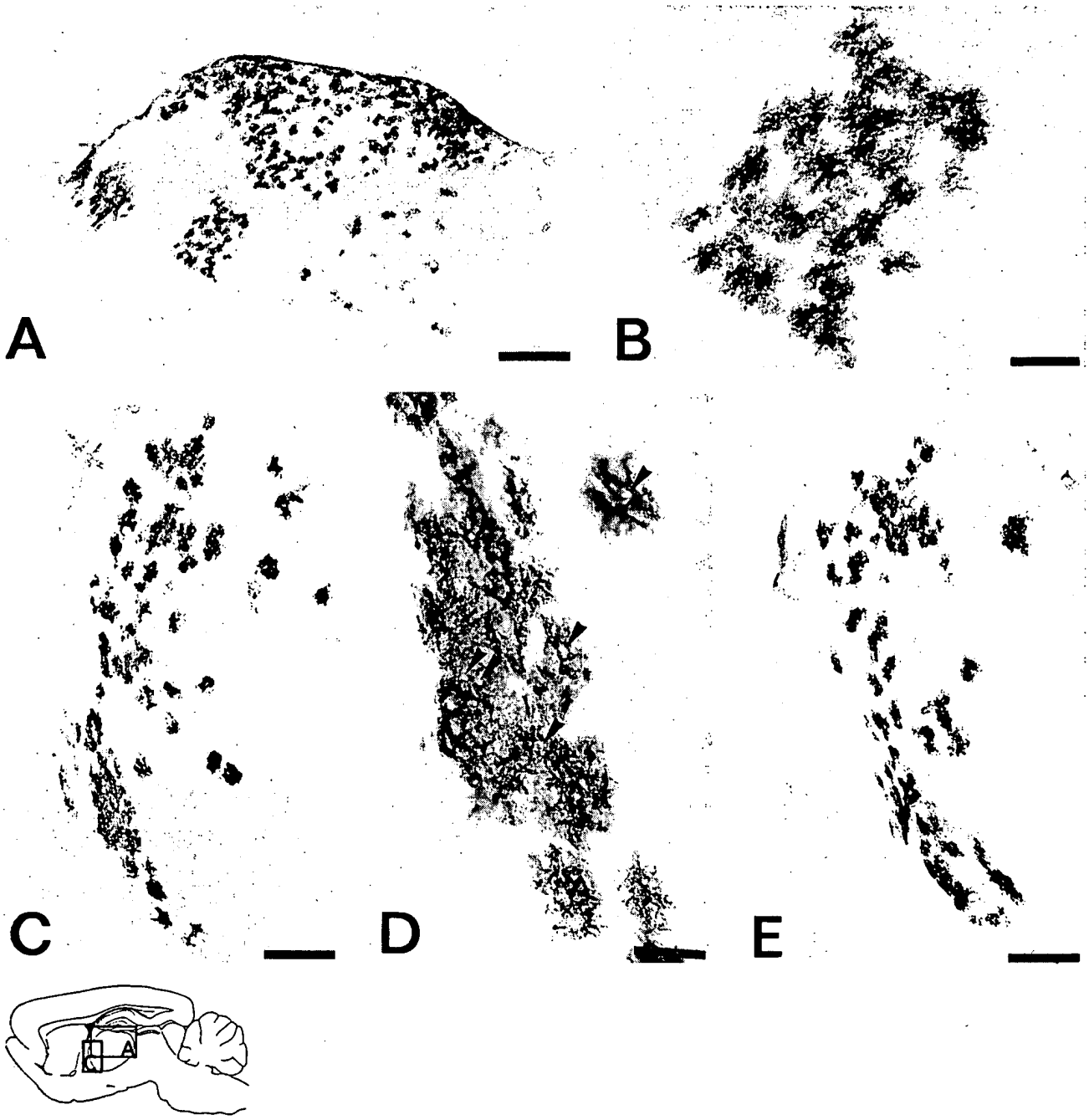
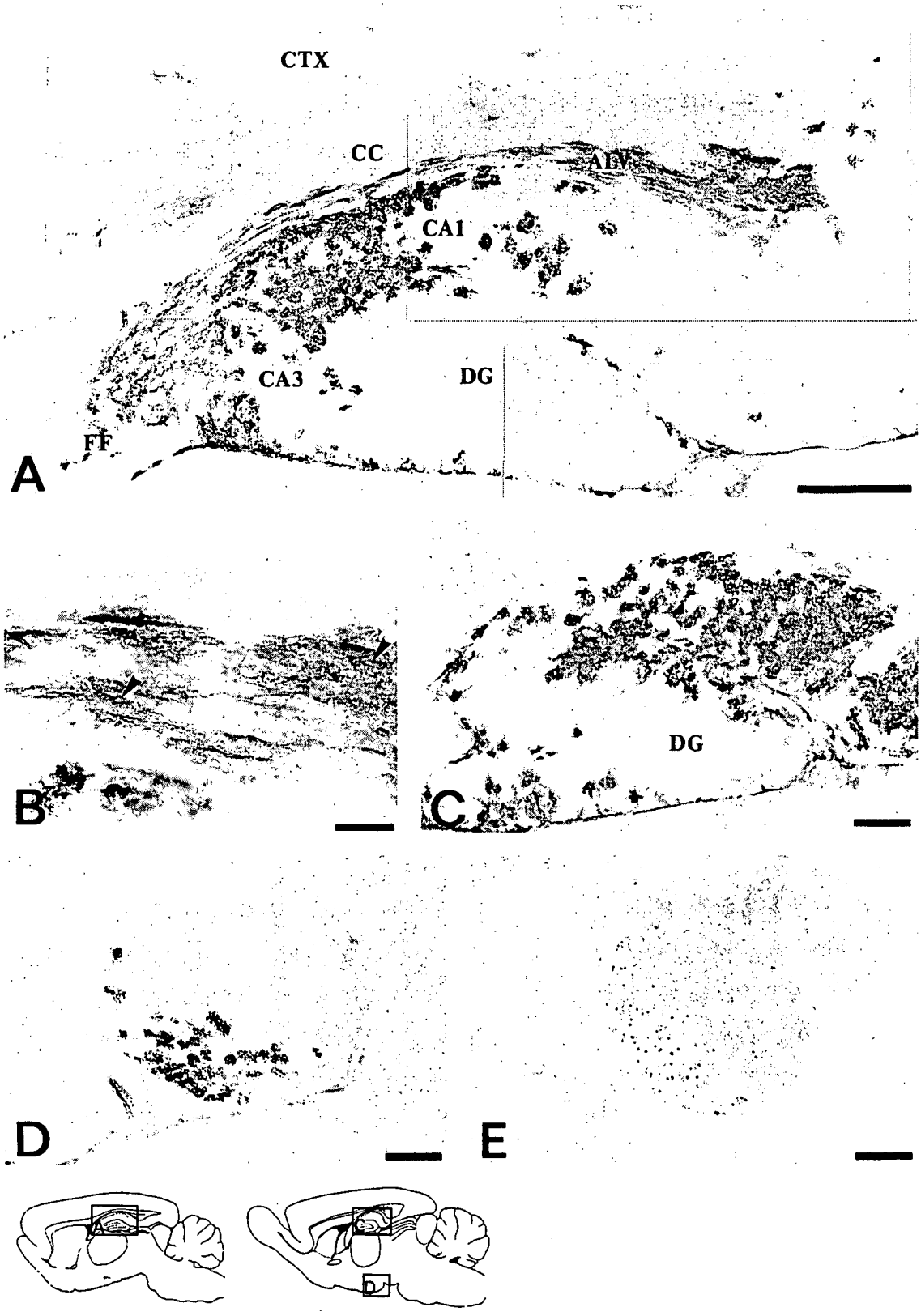


FIG. 4. Incorporation of transplanted cells in specific subnuclei of the thalamus. (A) Sagittal section showing widespread incorporation into the thalamus as revealed by the mouse-specific glial marker M2. (B) High-power view of one of the cell clusters in A. (C) M2 staining in the reticular thalamic nucleus, in which incorporation was frequently observed. (D) High-power view of cells integrated into the parafascicular thalamic nucleus. (E) M6 staining in a section adjacent to C. M6 and M2 stainings are overlapping and quite similar. The cellular processes and the membrane of the cell bodies are stained, while the cytoplasm is unstained giving the impression of holes in the M2- or M6-positive tissue. The cell bodies are small and slightly oval shaped (arrowheads in D); the processes frequently ramify and radiate in all directions reminiscent of protoplasmic grey matter astrocytes . Scale bars: A, 500 μm; B, 125 μm; C, 250 μm; D, 60μm; and E, 250 μm.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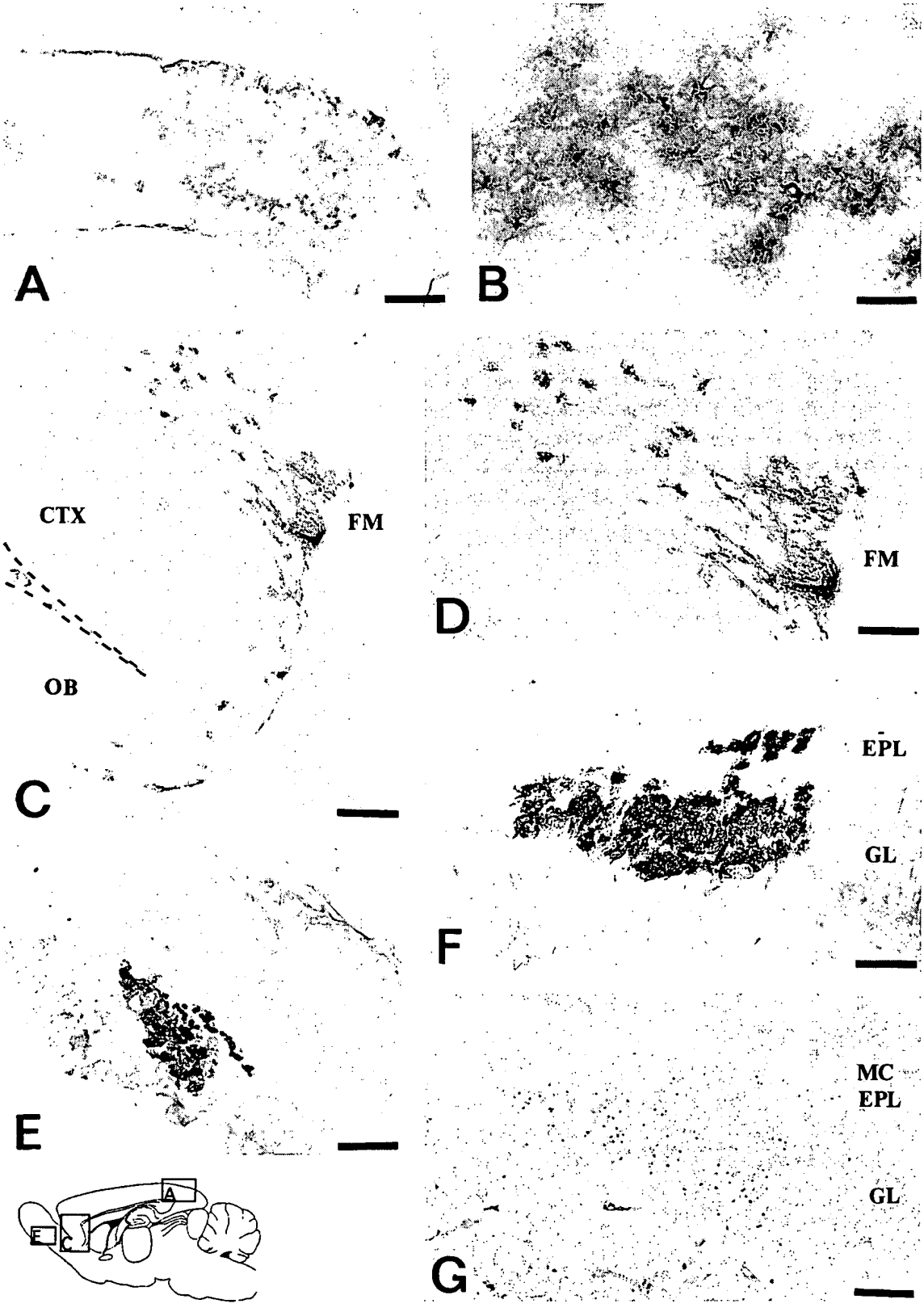
cells with predominantly glial-like morphology. All surviving cells were found within the host brain parenchyma. As previous data using intrauterine transplants of primary embryonic brain tissue indicate that at this host age proliferative cells exhibit an ability to incorporate into the host brain whereas postmitotic cells will remain as clumps within the ventricular space or attached to the ventricular walls (Cattaneo *et al.*, 1994; Campbell *et al.*, 1995; Fishell, 1995), this underscores the proliferative and non-postmitotic state of the EGF-responsive cells at the time of transplantation.

The pattern of incorporation of the EGF-responsive neural progenitors was markedly different from that previously obtained with intrauterine transplants of primary cell preparations obtained from the same embryonic brain regions (which will contain a mixture of precursors at different stages of development; see below). In particular, EGF-responsive progenitors derived from different rostrocaudal levels of the developing brain (cortex, striatum, or midbrain) displayed very similar patterns of incorporation without any preference for the areas from which they were derived. When primary telencephalic cells (from E14 to E16 cortex or striatum) are grafted in the same way, one portion of the injected cells shows widespread incorporation, but another subpopulation of the injected precursors displays region-specific incorporation with a preference for the homotopic brain region (Brüstle *et al.*, 1995; Campbell *et al.*, 1995; Fishell, 1995; Olsson *et al.*, 1998). Furthermore, primary cells derived from more caudal brain regions, such as the E12 ventral mesencephalon, were found to incorporate selectively into caudal brain structures, but not into the telencephalon (Campbell *et al.*, 1995; Olsson *et al.*, 1998). Differential adhesive properties of diverse precursor cell populations may explain these differences since removal of Ca^{2+}-dependent and -independent cellular adhesion molecules (by pretreatment of the cells with trypsin) changed the region-specific incorporation toward a widespread pattern (Olsson *et al.*, 1998). The present data, therefore, suggest that the EGF-responsive progenitor cell population studied here does not express the type of region-specific cell surface properties that are required for preferential incorporation and migration into the homotypic brain region.

The EGF-responsive progenitors differentiated into glial-like cells only, but not into neurons, after reintroduction into the embryonic brain environment. This is in contrast to the *in vitro* data showing that the EGF-responsive progenitors can differentiate into both neurons and glia (Reynolds *et al.*, 1992; Vescovi *et al.*, 1993; Svendsen *et al.*, 1995; Reynolds and Weiss, 1996; Carpenter *et al.*, 1997). It is also in contrast to the *in vivo* differentiation of primary telencephalic or mesencephalic precursor cells, similarly grafted into the embryonic forebrain ventricle, where site-specific development into both neurons and glia has been obtained (Brüstle *et al.*, 1995; Campbell *et al.*, 1995; Fishell, 1995). The stage of commitment of the cells at the time of transplantation, as well as the ability of the cells to respond to differentiating signals present in the developing host brain environment, may explain these differences. Differentiation of the EGF-responsive progenitors into astrocytes may thus represent a default pathway in the absence of appropriate differentiating cues. Interestingly, when the same EGF-responsive cells were transplanted into a myelin-deficient environment, remyelination and formation of large numbers of oligodendrocytes were observed (Hammang *et al.*, 1997).

Reynolds and Weiss (1992, 1996) have estimated that only between 0.1 and 1% of the dissociated E14 mouse ganglionic eminence cells proliferate in response to EGF. During cell culture, the number of multipotent progenitor cells increases by symmetrical divisions to approximately 25% (Reynolds and Weiss, 1996). Thus, the cultured spheres are likely to consist of a heterogenic mixture of stem cells and progenitor cells, but not postmitotic cells. When the EGF-dependent spheres are permitted to differentiate in culture they give rise to mainly glial cells, whereas only a smaller fraction (5–7% according to the present estimate) develops into neurons (Von Visger *et al.*, 1994; Ahmed *et al.*, 1995; Svendsen *et al.*, 1995; Rosser *et al.*, 1997). This raises the possibility that during cell culture, a portion of the cells present within the spheres has entered a phase of lineage restriction, perhaps committed to a glial fate. By contrast, the preparations of E14–E16 primary cells (as used for transplantation by Brüstle *et al.*, 1995; Campbell *et al.*, 1995; and Fishell, 1995) contain a mixture of

FIG. 5. Different types of glial cells in grey and white matter. (A) In the hippocampus M2-positive cellular profiles with morphological features of astrocytes were observed in all layers, while the dentate gyrus was consistently devoid of transplanted cells. (B) High-power view of A showing M2-positive cellular profiles present in the alveus. These cells possessed small oval cell bodies (arrowheads) with processes oriented along the myelinated fiber bundles, reminiscent of type II oligodendrocytes. (C) M2 staining in the hippocampus in a sagittal section more medial than that in A (see inset). (D) M2 staining in the mamillary body of the hypothalamus. (E) Distribution of nuclei of transplanted mouse cells stained by *in situ* hybridization for mouse satellite DNA in a section adjacent to D. (ALV, alveus hippocampi; CC, corpus callosum; CTX, cortex; DG, dentate gyrus; FF, fimbria fornix.) Scale bars: A, 500 μm; B, 60 μm; and C–E, 250 μm.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postmitotic cells and lineage-restricted progenitors, committed to an either neuronal or glial fate, whereas the numbers of nonrestricted multipotent progenitors is low, as suggested by *in vitro* and *in vivo* studies (Reynolds *et al.*, 1992; Grove *et al.*, 1993; Luskin *et al.*, 1993; Walsh and Cepko, 1993; Reid *et al.*, 1995). Furthermore, as neurogenesis is already well under way at the time point of tissue preparation (Berry and Rogers, 1965; Smart and Sturrock, 1979; Fentress *et al.*, 1981; Specht *et al.*, 1981; Marchand and Poirier, 1983; Bayer, 1984; Marchand and Lajoie, 1986; Levitt *et al.*, 1993), the number of cells committed to a neuronal fate is probably much higher than in the EGF-dependent cell culture.

Thus, the formation of both neurons and glia from primary brain precursors after intrautrine grafting may be due to the presence of progenitors that are lineage-restricted toward either a glial or a neuronal fate. These already committed precursors might receive the signals or factors that are necessary for their further differentiation. In the case of the EGF-responsive progenitors, the embryonic brain environment, which the cells were exposed to in the present experiments, may not fulfill all necessary requirements to induce the multipotent cells present in the grafted cell population to form neurons. Since neuronal differentiation from multipotent progenitors or stem cells is likely to be a complex multistep process involving different growth factors and expression of specific receptors (Cattaneo and McKay, 1990; Nurcombe *et al.*, 1993; Price, 1994; Goldman, 1995), the EGF-responsive progenitors, both *in vitro* and *in vivo*, may exhibit gliogenesis by default, in the absence of some critical differentiating signals.

An alternative possibility is that all surviving cells in the present experiment were derived from lineage-restricted precursors present in the cultured spheres and that the population of multipotent progenitors failed to survive, either because they were unable to pass across the ventricular wall or because they did not receive the appropriate survival factors. If so, the absence of neurons in the present experiment would be explained by the failure of the multipotent progenitors to become incorporated into the host brain tissue in this experimental paradigm. To further elucidate the role of multipotent cells for brain development, the identification of cell surface molecules specific for multipotent neural stem/progenitor cells, similar to the identification of hematopoietic stem cells (Weissmann, 1997), will be necessary. So far, the neural progenitor marker nestin (Lendahl *et al.*, 1990) has been identified, but its use is limited by the fact that it is present in lineage-restricted progenitors. The type of cells isolated with the cell culture system used here will have to be further characterized since both cell culture conditions and the age of the donor might effect the relative proportion of multipotent cells and lineage-restricted progenitors present in the cultured spheres (Svendsen *et al.*, 1995; Williams and Price, 1995). Interestingly, Kilpatrick and Bartlett (1995) have reported that isolation and growth of cells from older embryos than the ones used here (E17) give rise to a population of glial-restricted progenitors under EGF stimulation, while cells grown in the presence of bFGF have multipotent properties. Moreover, Suhonen *et al.* (1996) have recently shown that bFGF-dependent progenitors isolated from the adult brain can develop into both neurons and glia when transplanted to neurogenic areas (hippocampus or olfactory bulb) in adult recipients. Modifications of the culture conditions, the age from which the cells are isolated, and the nature of the environment into which the cells are grafted may all be important factors in determining phenotypic differentiation of progenitor cells after intracerebral transplantation.

In conclusion, the present study shows that CNS-derived neural progenitors, propagated under EGF stimulation for several weeks *in vitro*, can become incorporated into the developing brain when reintroduced into the embryonic brain environment by intrauterine transplantation. The results suggest that the cells isolated in this way have similar *in vivo* properties regardless of the embryonic brain region from which

FIG. 6. Migration routes of the transplanted cells toward the cortex and olfactory bulb. (A) Incorporation of the progenitor cells into the cortex as seen by M2 staining in a sagittal section. No preference for specific cortical layers was observed and cells were found in both deep and superficial layers. (B) High-power view of A, showing astrocyte-like cellular profiles with very thin processes. (C and D) M2-positive cellular profiles could be traced along the forceps minor (FM) into the frontal cortex (CTX) and along the so-called olfactocortical tract into the olfactory bulb (OB), suggesting possible migratory routes for the grafted cells after they had passed the ventricular wall. (D) High-power view of C, showing the orientation of the M2-positive grafted cells along the forceps minor (FM). (E) M2 staining in the olfactory bulb. The cells were very often seen to aggregate in clusters. (F) High-power view of M2 staining in the olfactory bulb. The cells were always located in the glomerular layer (GL) or in the external plexiform layer (EPL), but never in the mitral cell layer (MC) or subjacent layers. (G) *In situ* hybridization for mouse satellite DNA on a section adjacent to F showing that the grafted mouse cells were exclusively located in areas of M2 (or M6) staining. (CTX, cortex; EPL, external plexiform layer; FM, forceps minor; GL, glomerular layer; MC, mitral cell layer; OB, olfactory bulb.) Scale bars: A, 500 μm; B, 60 μm; C, 650 μm; D, 250 μm; E, 500 μm; and F and G, 250 μm.

they are derived and that the pattern of incorporation shows no preference for homotypic sites (striatum, cortex, or midbrain). The ability of the cells to develop into glia indicates that the immature progenitors can undergo differentiation either by default or in response to environmental cues present in the developing host brain. The results show that EGF-responsive neural progenitor cells may provide an interesting tool to understand the processes of brain development and cellular diversity. Their interesting *in vivo* properties, and their ability to become incorporated into the host brain, suggest moreover that they may be useful for cell-based therapies in the CNS.

EXPERIMENTAL METHODS

Cell Culture

Donor tissue was obtained from three different brain regions of E14 mouse embryos of timed pregnant CD1 mice (BK Universal, Stockholm, Sweden): (1) the ganglionic eminences (GE) including both the lateral and the medial ganglionic eminence, (2) a central portion of the frontoparietal cortical primordium (CTX), and (3) the ventral mesencephalon (VM). Pieces from 20 embryos were mechanically triturated into a single-cell suspension. The cells were plated at a density of 100,000 cells/ml in T75 culture flasks containing a serum-free medium composed of a 1:1 mixture of DMEM and F12 (Gibco) supplemented with epidermal growth factor (EGF; 20 ng/ml; human recombinant; R & D Systems) and a defined hormone and salt mixture including insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM), and sodium selenite (30 nM) (all from Sigma).

For further transplantation experiments transgenic mice carrying the lacZ transgene under control of different promoters were used: GFAP-lacZ transgenic mice (Brenner *et al.*, 1994; kind gift of Dr. Albee Messing, University of Wisconsin, Madison, WI), MBP-lacZ transgenic mice (Wrabetz *et al.*, 1998; kind gift of Dr. Lawrence Wrabetz, San Raffaele Scientific Institute, Milano), and nestin-lacZ transgenic mice (Zimmermann *et al.*, 1994; kind gift of Dr. Urban Lendahl, Karolinska Institute, Stockholm, Sweden). The ganglionic eminences of E14 timed pregnant transgenic mice were dissected and cell cultures were established from each striatum separately. Expression of the nestin-lacZ transgene was assayed by X-gal histochemistry of the remaining brain tissue. The tissue was permeabilized in wash buffer containing 0.1 M phosphate buffer (PB, pH 7.4), 2 mM $MgCl_2$, and 0.02% Nonidet P-40, followed by incubation in 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 1 mg/ml X-gal in wash buffer at 37°C for 2 h. Only cells derived from fetuses with transgene incorporation, i.e., the remaining brain tissue stained blue in the X-gal histochemistry, were used. To determine incorporation of the GFAP and MBP-lac Z transgenes into the fetal mouse genome, PCR was performed using unpurified DNA extracted from the remaining brain tissue, as described previously (Hanley and Merlie, 1991). Only cell lines established from transgenic fetuses were used in these experiments.

Every 7 days, the free-floating spheres of precursor cells were centrifuged and mechanically dissociated to a single-cell suspension using a fire-polished Pasteur pipet followed by resuspension in new T75 flasks at a density of 50,000 cells/ml. This passaging procedure was performed in total five times prior to transplantation.

For *in vitro* differentiation, the stem cells were plated as single cells or as clusters on polyornithine-coated glass coverslips. EGF was removed from the cell culture medium and replaced by 1% fetal bovine serum. After 5–10 days *in vitro* cells were fixed for 10 min with 4% paraformaldehyde (PFA), rinsed three times in 0.1 M PB, and processed for immunocytochemistry.

In Utero Transplantation

For transplantation, the progenitor cells were collected in DMEM/F12 3–4 days after the last passage when they had started to form small spheres of 5–20 cells. This was done to ensure high cell viability during transplantation; pilot tests indicated that cell viability monitored by trypan blue dye exclusion was reduced in the single-cell suspensions several hours after collection. The mouse precursor cells were injected into the ventricle of age-matched E15 rat embryos *in utero* as previously described (Campbell *et al.*, 1995). Timed pregnant Sprague–Dawley rats (BK Universal, Stockholm, Sweden) were anaesthetized with halothane (1.5% halothane/air mixture) and a midline laparotomy was performed. The uterine horns were removed separately from the abdominal cavity and each embryo was oriented with help of transillumination so that the forebrain ventricles were visible. One microliter of cell suspension containing approximately 100,000 cells derived from GE, CTX, or VM was injected freehand into the ventricle by one experimenter using a glass capillary (outer diameter: 50–70 μm) connected to a 10-μl Hamilton syringe (Nikkhah *et al.*, 1994) while a second experimenter held the embryo in place. After transplantation the uterine horns were placed back into the abdomen, and the animal was sutured and left to give

birth. About 60% of the transplanted embryos were born and of those about 60% contained surviving transplants.

At 4 weeks after birth, the rat pups were deeply anesthetized with chloral hydrate and perfused through the ascending aorta with 0.9% saline followed by ice-cold 4% PFA in 0.1 M PB. After postfixation overnight in PFA and dehydration in 20% sucrose/0.1 M PB, sagittal sections were cut at 30 µm thickness on a freezing microtome. Series of sections were processed for immunohistochemistry.

Antibodies

The primary antibodies used were mouse monoclonal antibodies to β-galactosidase (1:500; Sigma), β-tubulin (1:1000; Sigma), GFAP (1:500; Boehringer), O4 (1:25; Boehringer-Mannheim), S-100β (1:1000; Sigma), and vimentin (1:25; DAKO); rat monoclonal antibodies to M2 and M6 (1:50; kind gift of Dr. C. Lagenaur); and rabbit polyclonal antibodies to myelin basic protein (MBP; 1:250; Zymed), myelin proteolipid protein (PLP; 1:250; Serotec), and nestin (1:1000; kind gift of Dr. R. D. G. McKay). Secondary antibodies were FITC-conjugated and TRITC-conjugated antibodies to mouse, rat, or rabbit IgG (1:100; Sigma) or biotinylated antibodies to mouse, rat, or rabbit IgG (1:225; Vector).

Immunocytochemistry

For double-fluorescent immunocytochemistry, cultures were preincubated in 1% of the appropriate sera and 1% Triton-X 100 (Sigma) in 0.1 M PB for 1 h at room temperature. Following a 2-h incubation with the primary antibodies at room temperature, the cultures were rinsed three times and incubated with the FITC- and TRITC-conjugated antibodies for 30 min at room temperature in the dark.

For triple immunocytochemistry for GFAP, PLP, and the GFAP-lacZ reporter gene, cultures were exposed to the X-gal histochemistry overnight at 33°C (blue reaction product). The next day cultures were reacted with a mouse monoclonal antibody to GFAP, followed by the mouse ABC Elite kit and labeling with AEC (red reaction product). After extensive washing cultures were reacted with a rabbit polyclonal antibody to PLP, followed by the rabbit ABC Elite system and labeling with 3,3-diaminobenzidine (DAB, 0.5 mg/ml, Sigma) with nickel intensification (black reaction product).

Immunohistochemistry

Series of sections were stained for M2 (Lund *et al.*, 1989), M6 (Lund *et al.*, 1985), nestin, and vimentin. In addition, sections from transgenic animals were stained for X-gal histochemistry or β-galactosidase to detect transgene expression. In brief, following a 10-min incubation in 3% H_2O_2/10% methanol and a 1-h preincubation in the appropriate serum, sections were incubated overnight in the primary antibody at room temperature. After three rinses with PB, sections were incubated in the appropriate biotinylated secondary antibody for 1 h, rinsed three times, and transferred to a Vectastain ABC solution for 1 h. DAB in 0.01% H_2O_2 served as the chromogen in the subsequent visualization reaction. Sections were mounted on chromalum-coated slides, dehydrated in ascending alcohol concentrations, cleared in xylene, and coverslipped in DPX. In addition, selected sections were processed for double-fluorescent immunohistochemistry with M2 and either GFAP or S-100β.

In Situ Hybridization

Another series of sections was processed for *in situ* hybridization for mouse satellite DNA as previously described (Brüstle *et al.*, 1995). The sections were mounted on chromalum-coated slides, left to dry, and then fixed with 4 % PFA. After a 30-min preincubation in 2× SSC and 5 mM EDTA at 37°C, the sections were digested with protease from *Streptomyceus griseus* (Sigma; 25 µg/ml) in 2× SSC and 5 mM EDTA (pH 8.0) for 10 min at 37°C. The sections were dehydrated in ascending ethanols and denatured in 70% formamide (2× SSC) for 10 min at 90°C. Following additional dehydration with ice-cold ethanols, hybridization was carried out overnight at 37°C with a digoxigenin end-labeled oligonucleotide probe to mouse satellite DNA (Hörz and Altenburger, 1981; 15 ng/section, in 50% formamide, 4× SSC, 1× Denhardt's solution, 10% dextran sulfate, and 500 µg/ml salmon sperm DNA at pH 7.0). After two 30-min washes in 2× SSC and 0.1× SSC at 37°C and cooling to room temperature, labeled nuclei were visualized using alkaline phosphatase detection: Following preincubation in 2% normal goat serum (NGS)/0.2% Triton X-100 in buffer A (100 mM Tris–HCl, 150 mM NaCl, pH 7.5), sections were incubated for 4 h in an alkaline phosphatase-conjugated antibody to digoxigenin (1:500, Boehringer-Mannheim) in 1% NGS/0.1% Triton X-100/buffer A. After three rinses with buffer A and a 2-min incubation in buffer B (100 mM Tris–HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5), labeled nuclei were visualized with 4-nitroblue tetrazolium (NBT; 340 µg/ml, Boehringer-Mannheim) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; 170 µg/ml, Boehringer-Mannheim) in buffer B for 4 h in the dark.

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The rising star of neural stem cell research

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A neuroscientist, gazing up at the night sky full of glittering stars—some of them bright and very close, others more distant and barely visible—cannot help but be struck by the similarity to brain sciences. Like stars, many aspects of neuroscience are clearly discernible, whereas others remain hidden from sight, beyond the realm of current knowledge. Ultimately, it will be necessary to piece together all this information to obtain an accurate picture of the nervous system and its inner workings. In this context, the rapidly growing field of neural progenitor and stem cell biology promises to become an increasingly bright star in the coming years. Two papers in this issue[1,2] provide a hint of the potential of neural stem cell approaches in replacement therapy and as candidates for central nervous system (CNS) gene therapy paradigms.

Over the years, enormous attention has focused on understanding the developmental origins of the nervous system. Scientists have postulated the existence of a single "stem" cell—a mother or queen of all cells—that is self-renewable and multipotent (i.e., capable of generating various committed progenitor cells and ultimately differentiating into mature cells)[3]. A neural stem cell (NSC) is defined as a single cell with the ability to proliferate, exhibit self-maintenance or renewal over the lifetime of the organism, generate a large number of clonally related progeny, retain its multilineage potential over time, and produce new cells in response to injury or disease[4]. This last criterion has proved particularly troublesome for neurobiologists.

As it is difficult to determine whether a cell within the brain can display all of the features listed above, investigators usually prefer to use terms like "putative stem cells," "stem-like cells," or "multipotent progenitors" to define mitotic cells that do not seem to be terminally differentiated and can give rise to cells of multiple neural lineages. The interest in neural stem cell biology derives, however, not only from its importance in understanding neural development, but also in its potential for providing therapies to combat neurodegenerative disease.

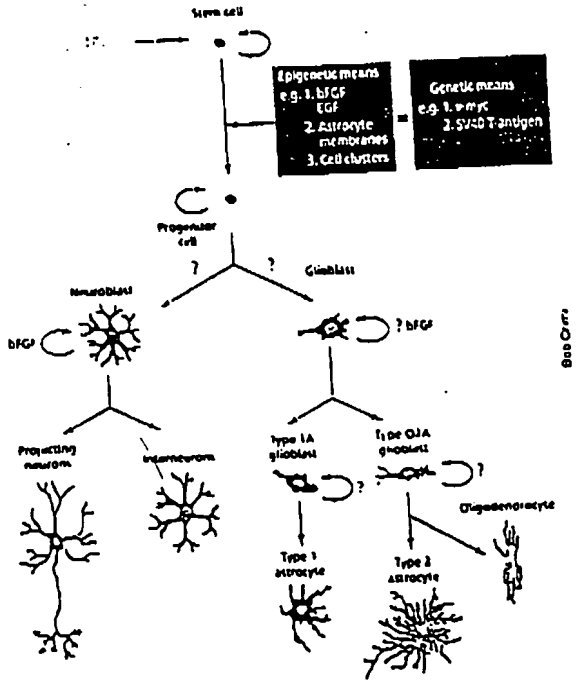

Figure 1. Lineage of neural stem cells present in fetal brain. A single stem cell capable of extended self-renewal (indicated by the arrow that loops around the top of the cell), gives rise to progenitor cells that then generate neuroblasts (neuronal precursors) or glioblasts (glia precursors). These precursors give rise to different types of neurons and glia. Factors that influence cells at particular stage of differentiation as well as epigenetic and genetic means of propagation (Snyder's study) have been indicated. Figure adopted from ref. 10.

Until now, almost all our knowledge about the properties of NSCs has been based on studies using cells originating from embryonic, neonatal, or adult rodent CNS. The report by Evan Snyder and his collaborators[1] in this issue provides strong evidence that human NSCs are able to perform in vitro and in vivo all the critical functions previously described for their rodent counterparts. The authors show that the clones of neural cells isolated from ventricular zone of the human fetal telencephalon, particularly the ventricular zone, can be safely and equally effectively propagated by either epigenetic (basic fibroblast growth factor; bFGF) or genetic (constitutively downregulated v-*myc*) means. Importantly, these clones behave identically. After plating into serum-containing medium, they differentiate spontaneously into neurons and glia, thus fulfilling the requirements of multipotency.

In addition to cells expressing the variety of differentiated lineage-specific markers, each clone also contained new immature cells, which could be passaged again. These cells could give rise to a new population of clonally related cells, some of them expressing a variety of differentiated neural markers of multiple neural lineages, others expressing the marker of immature cells (vimentin), which are suitable for another passage, thus securing the self-renewability of multipotent clones (see Fig. 1).

To assess the potential of these cells as a vehicle for molecular therapies, Snyder and his colleagues evaluated the ability of human NSCs to correct a prototypical genetic defect in an in vitro model; the defect chosen was that underlying Tay–Sachs disease, in which the pathological accumulation of GM2 gan-

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glioside in the brain leads to progressive neurodegeneration. In their experiments, the metabolic defect in primary neural cultures could be corrected by human NSCs. The efficacy observed suggests the feasibility of using human NSCs to supply a variety of therapeutic gene products to abnormal neural cells in disease.

Snyder and his colleagues subsequently implanted the human NSC clones into the lateral ventricle of newborn mice, where they integrated into the subventricular zone. From this region, they migrated extensively, either along the subcortical white matter or along the rostral migratory stream, and differentiated into cell types developmentally appropriate for the time and region of the implantation: Oligodendrocytes/astrocytes in cortical and subcortical regions and neurons in the olfactory bulb, respectively. When implanted into the cerebellum at the opposite end of the neuraxis, they yielded different neuronal cell types, mainly cerebellar granule cells.

In addition, the human NSC clones were able to "read" the developmental cues operating in different regions of the neonatal rat brain, and after completing their migration, expressed the phenotype of one of the three fundamental neural lineages. Importantly, clones engineered ex vivo by a retroviral vector to express an exogenous gene could express that gene in vivo, further establishing their efficacy for molecular gene therapy.

Snyder's group also studied the effectiveness of human NSCs to replace neurons in a neurological mouse mutant of cell deficiency (somewhat like "ablation"). In the meander tail (*mea*) mutant, characterized by a failure of granule neurons to develop and survive in cerebellum[3], engrafted clones of human NSCs were able to replace missing neurons and intermix with residual endogenous murine host granule neurons. The plasticity of human NSCs to respond to certain local cues is even more impressive as they were not harvested from the postnatal brain or cerebellum, but from the periventricular region of the fetal telencephalon, an area which presumably does not normally give rise to cerebellar granule neurons.

Important and complementing observations on the properties of human progenitor cells are outlined in an accompanying article by Ron McKay and colleagues[5]. In their study, fetal human donor cells, albeit of unknown clonal relationship, are deposited in the cerebral ventricles of embryonic rats, allowing them free access to large areas of the neuroepithelium. One to eight weeks after transplantation, recipients of acutely dissociated and bFGF/epidermal growth factor-treated preparations showed incorporated cells in a variety of grey and white matter regions, where they differentiated into all three major cell types. This incorporation pattern—previously described after intrauterine transplantation of rodent cells[6]—suggests that donor cell migration is not primarily determined by cell-autonomous properties, but rather by guidance cues within the host brain. Responsiveness of human donor cells to migration cues within a rodent brain implies remarkable conservation of these signals across species.

Of particular importance is their finding that transplanted human cells are able to replace large areas of the subventricular zone, which is known to serve as an endogeneous source of multipotential neural precursors giving rise to neurons and glia throughout life[7]. Thus, the incorporation of human neural precursors into the rat subventricular zone offers interesting insights into future cell replacement strategies.

Another important aspect of the study by McKay and colleagues is the finding that numerous human cells populating the white matter throughout the brain acquire an oligodendroglial phenotype and participate in the myelination of host axons. In the future, this model could be used and adapted in various ways to study the mechanism of myelin repair in human demyelinating diseases, whereby widespread delivery of oligodendrocytes would be desirable.

A glance at the literature reveals the frequency with which the term "stem cell" has been used rather loosely and inappropriately[8]. Snyder's study emphasizes clonality, the key for NSC definition, allowing an assessment of the true potential of individual cells. This will be an even more urgent requirement when it comes to therapeutic applications. The choice of the propagation technique, epigenetic or genetic, will depend on particular research or clinical problems. On the basis of the results of the Snyder paper, they are equally safe and effective. Yet both these approaches warrant careful scrutiny before going into humans. Knowing that both methods are giving comparable results will allow the pooling of data from various laboratories in order to apply them to human clinical neurobiology, with the hope that one day, the NSC could become the brightest of all stars in the new millennium of neuroscience and brain repair.

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Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes

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Stable clones of neural stem cells (NSCs) have been isolated from the human fetal telencephalon. These self-renewing clones give rise to all fundamental neural lineages in vitro. Following transplantation into germinal zones of the newborn mouse brain they participate in aspects of normal development, including migration along established migratory pathways to disseminated central nervous system regions, differentiation into multiple developmentally and regionally appropriate cell types, and nondisruptive interspersion with host progenitors and their progeny. These human NSCs can be genetically engineered and are capable of expressing foreign transgenes in vivo. Supporting their gene therapy potential, secretory products from NSCs can correct a prototypical genetic metabolic defect in neurons and glia in vitro. The human NSCs can also replace specific deficient neuronal populations. Cryopreservable human NSCs may be propagated by both epigenetic and genetic means that are comparably safe and effective. By analogy to rodent NSCs, these observations may allow the development of NSC transplantation for a range of disorders.

Keywords: cell therapy, progenitor cell, gene therapy, Tay-Sachs disease, transplanation, differentiation

Neural stem cells (NSCs) are primordial, uncommitted cells postulated to give rise to the array of more specialized cells of the central nervous system (CNS)[1–5]. They are operationally defined by their ability (1) to differentiate into cells of all neural lineages (i.e., neurons—ideally of multiple subtypes, oligodendroglia, astroglia) in multiple regional and developmental contexts; (2) to self-renew (to give rise to new NSCs with similar potential); and (3) to populate developing and/or degenerating CNS regions. The demonstration of a monoclonal derivation of progeny is obligatory to the definition (i.e., a single cell must possess these attributes). With the earliest recognition that rodent neural cells with stem cell properties, propagated in culture, could be reimplanted into mammalian brain where they could reintegrate appropriately and stably express foreign genes[6–13], gene therapists and neurobiologists began to speculate how such a phenomenon might be harnessed for therapeutic advantage as well as for understanding developmental mechanisms. These, and the studies they spawned (reviewed in refs. 14–16), provided hope that the use of NSCs might circumvent some limitations of presently available graft material[14] and gene transfer vehicles[17] and make feasible a variety of therapeutic strategies.

Neural cells with stem cell properties have been isolated from the embryonic, neonatal, and adult rodent CNS and propagated in vitro by a variety of equally effective and safe means—both epigenetic (with mitogens such as epidermal growth factor [EGF][18] or basic fibroblast growth factor [bFGF][19,18,20] or with membrane substrates[21]) and genetic (with propagating genes[22] such as *v-myc*[18,20] or large T-antigen [*T-Ag*][23]). Maintaining NSCs in a proliferative state in culture does not subvert their ability to respond to normal developmental cues in vivo following transplantation (such as the ability to withdraw from the cell cycle, interact with host cells, and differentiate[24]). These extremely plastic cells migrate and differentiate in a temporally and regionally appropriate manner particularly following implantation into germinal zones. Intermingling nondisruptively with endogenous progenitors, responding similarly to local cues for their phenotypic determination, and appropriately differentiating into diverse neuronal and glial types, they participate in normal development along the rodent neuraxis. In addition, they can express foreign genes in vivo[6–13], often in widely disseminated CNS regions[25,26], and are capable of neural cell replacement[7].

The presumption has been that the biology that endows such rodent cells with their therapeutic potential is conserved in the human CNS. If true, then progress toward human applications may be accelerated. We demonstrate the potential of clones of human NSCs to perform these critical functions in vitro and in vivo in a manner analogous to their rodent counterparts.

Results and discussion

Isolation, propagation, and cloning of human NSCs. The isolation, propagation, characterization, cloning, and transplantation of NSCs from the human CNS mirrored strategies used for the murine NSC clone C17.2 (propagated following transduction of a constitutively downregulated *v-myc*[18,20]) and for growth factor-expanded murine NSC clones[18]. NSCs—even genetically propagated clones[27]—require molecules like bFGF and/or EGF in serum-free medium to divide[18,28]. Therefore, this dual responsiveness was

chosen for both screening and enriching a starting population of stable, dissociated, cultured primary human neural tissue for cells. Cells dissociated from human fetal telencephalon—particularly the ventricular zone, which has been postulated to harbor (in lower mammals) a rich NSC population—were initially grown as a polyclonal population first in serum-supplemented and then in serum-free medium containing bFGF and/or EGF. Cells were transferred between media containing one or the other of the mitogens to select for dual responsiveness. Some populations were then maintained in bFGF alone for subsequent manipulation and cloning; others were used for retrovirally mediated transduction of *v-myc* and subsequent cloning.

To provide an unambiguous molecular tag for assessing the clonal relationships of the cells, as well as to facilitate identification of some cells following transplantation and to assess their capacity to express exogenous genes in vivo, some bFGF-propagated subpopulations were infected with an amphotropic replication-incompetent retroviral vector encoding *lacZ* (and *neo* for selection). Single resistant colonies were initially isolated by limiting dilution. Monoclonality of the cells in a given colony was then confirmed by demonstrating the presence of only one copy of the *lacZ/neo*-encoding retrovirus, with a unique chromosomal insertion site. In clone H1, for example, all *lacZ/neo*-positive cells, had a single, common retroviral integration site indicating that they were derived from a single infected "parent" cell (Fig. 1A).

In rodents, genes (such as *v-myc* and *T-Ag*) that interact with cell cycle regulatory proteins have been used to propagate NSCs[5,6], neural progenitors[3], and neuroblasts[12], resulting in engraftable rodent NSC clones that can be manipulated and have therapeutic potential[4]. Therefore, some of the bFGF-maintained human cell populations, enriched for NSCs, were infected with an amphotropic, replication-incompetent retroviral vector encoding *v-myc* and *neo*[10] yielding multiple colonies. All of the putative clones had only one unique retroviral insertion site, demonstrating their monoclonality (Fig. 1B). Five clones (H6, H9, D10, C2, and E11) were generated and maintained in serum-free medium containing bFGF.

Multipotency and self-renewal in vitro. In uncoated dishes and in serum-free medium supplemented with bFGF, all clones grew in culture as clusters that could be passaged weekly for at least 1 year (Fig. 2A). The cells within these clusters expressed vimentin, a neural progenitor marker[13]. By dissociating these clusters and plating them in serum-containing medium, these clones differentiated spontaneously into neurons and oligodendrocytes (Figs. 2B and C). After 5 days under these differentiating conditions, 90% of the cells in all clones became immunoreactive for the neuronal marker neurofilament (NF; Fig. 2B); 10% expressed CNPase, a marker for oligodendroglia (Fig. 2C). Mature astroglia containing glial fibrillary acidic protein (GFAP) were not initially observed, even after 1 month under these culture conditions. However, GFAP production could be induced by coculture with primary dissociated embryonic murine CNS tissue (Fig. 2D). In addition to cells expressing the variety of differentiated lineage-specific markers (establishing "multipotency"), each clone gave rise to new immature vimentin-positive cells (Fig. 2E), which could, upon subsequent passage, give rise to new cells expressing multiple differentiated neural markers as well as to new vimentin-positive passageable cells (i.e., "self-renewability"). All the clones, whether genetically modified or epigenetically maintained, were similar in vitro.

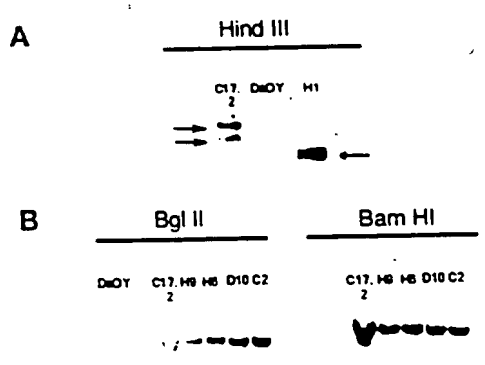

Figure 1. Southern blot analysis of retroviral insertion into human NSC clones. (A) Genomic DNA from clone H1 (propagated in bFGF and transduced with a retrovirus encoding *lacZ* and *neo*) digested with Hind III (cuts once within the provirus) and incubated with a radiolabeled *neo* probe. The murine NSC clone C17.2 contains two integrated proviruses encoding *neo*[10,20]. DaOY is an uninfected human meduloblastoma cell line. (B) Genomic DNA from clones H9, H6, D10, and C2 (propagated in bFGF and/or EGF and infected with a retrovirus encoding *v-myc*) were digested with BglII or BamHI (cuts once within the provirus) and probed for *v-myc*. C17.2 contains one *v-myc*-encoding provirus.

Ability to cross-correct a genetic defect. To assess their potential as vehicles for molecular therapies, we compared the ability of human NSCs to complement a prototypical genetic defect to murine NSCs[14]. The neurogenetic defect chosen was in the α-subunit of β-hexosaminidase, a mutation that leads to hexosaminidase-A deficiency and a failure to metabolize GM_2 ganglioside to GM_3 (Tay-Sachs disease [TSD]). Pathologic GM_2 accumulation in the brain leads to progressive neurodegeneration. The ability of human NSCs to cross-correct was compared with that of two established murine NSC clones: C17.2 and a subclone of C17.2 (C17.2H) engineered via retroviral transduction of the human α–subunit gene to overexpress hexosaminidase[15]. These murine NSC clones secrete functional hexosaminidase-A[15]. A transgenic mouse with an α-subunit deletion[16] permitted examination of the ability of human NSCs to secrete a gene product capable of rescuing TSD neural cells. NSCs (murine and human) were cocultured with dissociated TSD mouse brain cells from which they were separated by a porous membrane that allowed passage of hexosaminidase but not cells. After 10 days, the mutant neural cells were examined: (1) for the presence of hexosaminidase activity (Fig. 3A–C, and M); (2) with antibodies to the α-subunit and to CNS cell type markers to determine which TSD neural cells internalized corrective gene product (Fig. 3D–L); and (3) for reduction in GM_2 storage (Fig. 3N). While there was minimal intrinsic hexosaminidase activity in TSD cells cultured alone (Fig. 3A), hexosaminidase activity increased to normal intensity when the cells were cocultured with murine or human NSCs (Fig. 3B and C). The extent of human NSC-mediated cross-correction matched the success of murine NSCs, yielding percentages of hexosaminidase-positive TSD cells significantly greater than in untreated controls ($p<0.01$) (Fig. 3M). All neural cell types from the TSD mouse brain were corrected (Figs. 3D–L). The percentage of TSD CNS cells without abnormal GM_2 accumulation was significantly lower in those exposed to secretory products from human NSCs than in untreated TSD cultures ($p<0.01$), approaching those from wild-type mouse brain (Fig. 3N).

Multipotency and plasticity in vivo. We next determined whether human NSC clones (whether epigenetically or genetically propagated) could respond appropriately to normal developmental cues in vivo, which include migrating appropriately; integrating into host parenchyma; and differentiating into neural cell types appropriate to a given region's stage of development, even if that stage is not the one in which the NSCs were obtained. Although there are many approaches for testing these qualities[17–19], we used paradigms similar to those we have used with murine NSCs to assess their developmental ability[4]. When murine NSC clones are implanted into the cerebral ventricles of newborn mice, the cells engraft in the subventricular germinal zone (SVZ)[4] and follow the

established pathways used by endogenous progenitors, either migrating along the rostral migratory stream (RMS) to the olfactory bulb (OB), becoming neurons[9], or migrating into subcortical and cortical regions (where gliogenesis predominates and neurogenesis has ceased) becoming oligodendroglia and astroglia[10]. When transplanted into the germinal zone of the neonatal mouse cerebellum (the external germinal layer [EGL]), these same NSCs migrate inward and differentiate into granule neurons in the emerging internal granule cell layer (IGL)[11]. Following intraventricular implantation, human NSC clones emulated the developmentally appropriate behavior of their murine counterparts (Fig. 4 and 5). The engraftment, migration, and differentiation of epigenetically perpetuated clones were identical to that of *v-myc* perpetuated clones. Three of the five *v-myc* clones engrafted well (Table 1).

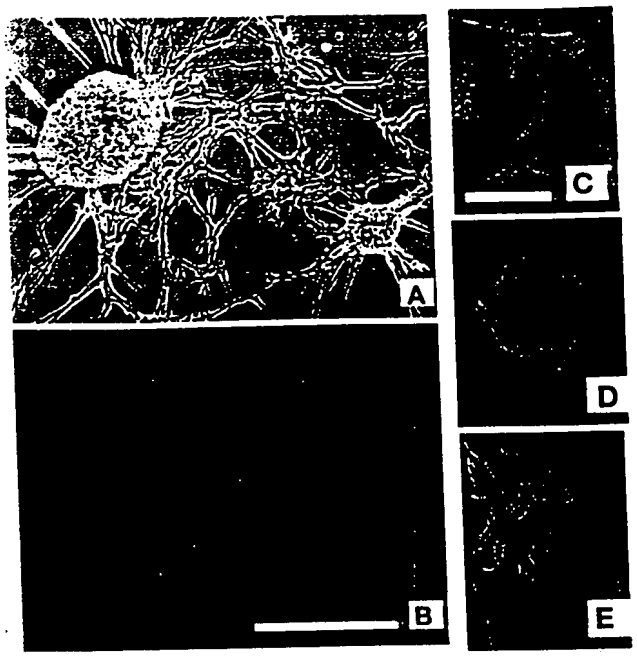

Figure 2. Characterization of human NSCs in vitro. (A) NSCs grown in serum-free medium. Immunostaining for (B) the neuronal marker neurofilament or (C) the oligodendroglia marker CNPase in serum-containing medium. (D) Immunostaining for the astrocyte marker human GFAP upon coculture with primary murine CNS cultures. (E) Immunostaining for the immature neural marker vimentin at transfer to serum-containing medium.

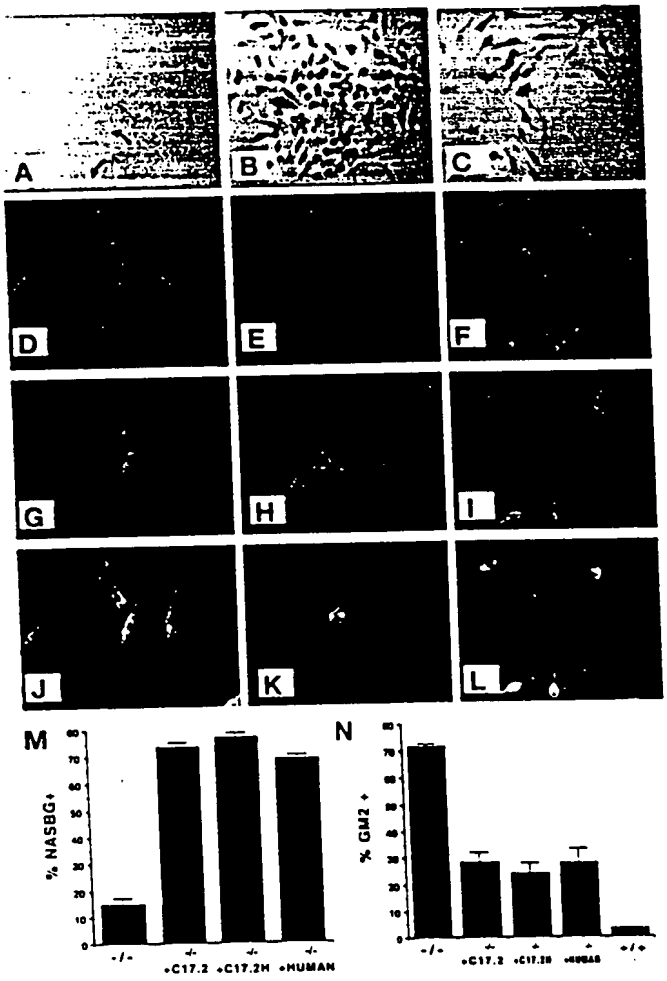

Figure 3. Dissociated brain cells from mice with mutated α–subunit of β-hexosaminidase (Tay-Sachs disease) cocultured with human NSCs. (A–C) Hexosaminidase activity determined by NASBG histochemistry. (A) TSD neural cells (arrows) not exposed to NSCs. TSD cells exposed to secretory products from (B) murine NSC clone C17.2H or from (C) human NSCs. (D–L) TSD cells cocultured with human NSCs immunostained with a (D–F) fluorescein-labeled antibody to the human α-subunit of β-hexosaminidase and (G–I) with antibodies to neural cell type-specific antigens. (G) Neuronal-specific NeuN marker; (H) glial specific GFAP marker; and (I) precursor maker, nestin. (J–L) Dual filter microscopy of the α-subunit and cell-type markers. (M) Percentage of β-hexosaminidase positive TSD cells; -/-: TSD α-subunit–null cells; TSD cells exposed to secretory products from C17.2+ murine NSCs; C17.2H+: murine NSC engineered to overexpress murine hexosaminidase; +human: human NSCs. (N) GM, accumulation in TSD cells; labels as in (M); +/+: wild-type mouse brain.

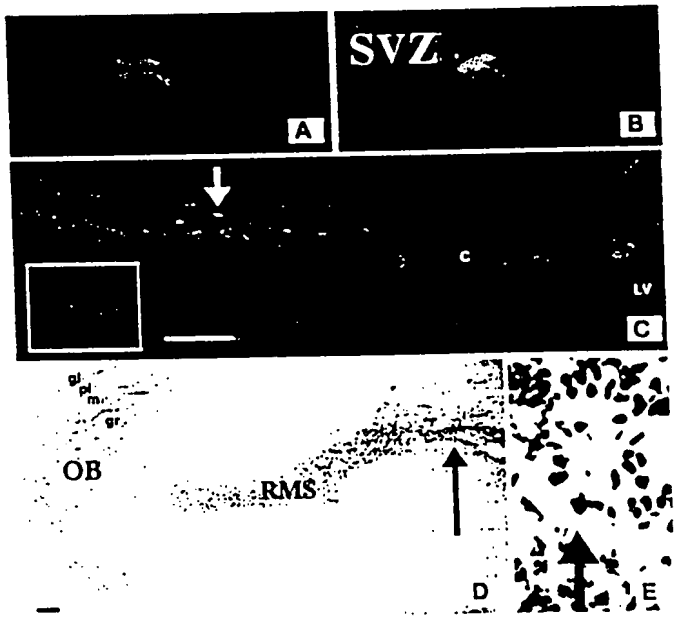

Figure 4. Migration of human NSCs following engraftment into the SVZ of newborn mice. (A,B) Human NSCs 24 h after transplantation. (A) Donor-derived cell (red) interspersed with (B) densely packed endogenous SVZ cells, visualized by DAPI (blue) in the merged image. (C) Donor-derived cells (red) within the subcortical white matter (arrow) and corpus callosum (c) and their site of implantation in the lateral ventricles (LV). Arrow indicates the cell shown at higher magnification within the inset. (D) Donor-derived cell migration from the SVZ into the rostral migratory stream (RMS) leading to the olfactory bulb (OB), in a cresyl-violet counterstained parasagittal section; gl: glomerular layer; pl: plexiform layer; m: mitral layer; gr: granular layer. Scale bars: 100 μm. (E) Higher magnification of area indicated by the arrow in (D). Brown staining indicates BrDU-immunoperoxidase-positive donor-derived cells.

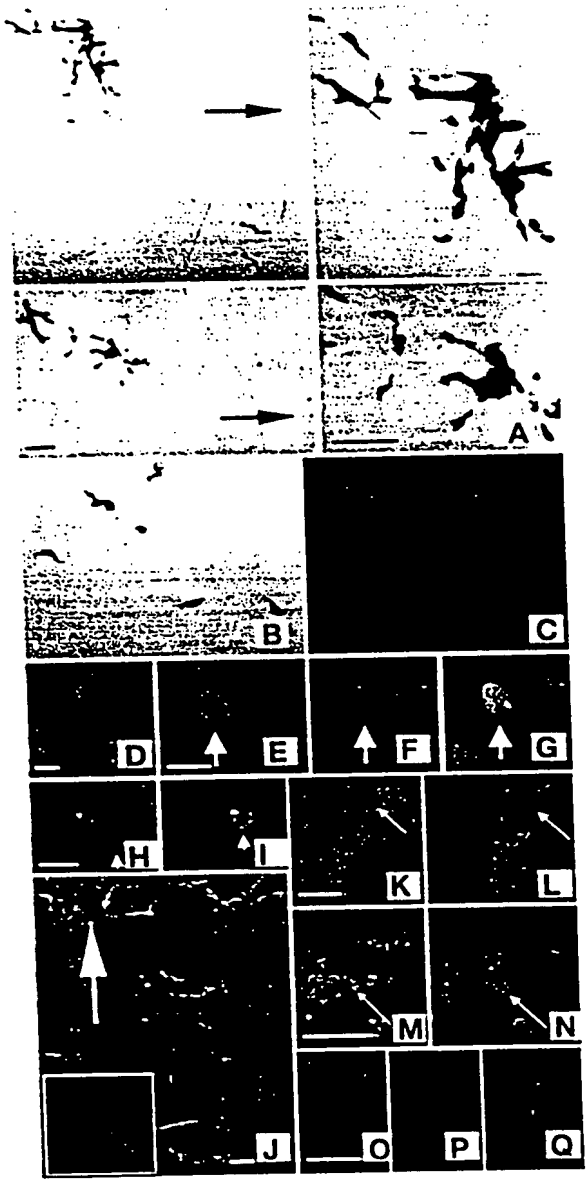

Figure 5. Characterization of human NSC clones in vivo following engraftment into SVZ of neonatal mice. (A–C) *LacZ*-expressing donor-derived cells from human NSC clone H1 detected with (A,B) Xgal and with (C) anti-β-galactosidase within (A) the periventricular and subcortical white matter regions and (B,C) OB granule layer. The arrows in (A) indicate the lateral ventricles. (D–G) BrdU-labeled NSCs (clone H6) implanted into the SVZ at birth identified in the OB with a (D) human-specific NF antibody and by (E–G) BrdU ICC via confocal microscopy. (E) BrdU-positive cell visualized by fluorescein; (F) anti-NeuN+ antibody visualized by Texas Red; (G) same cell visualized by dual filter. Donor-derived clone H6 in the adult subcortical white matter double-labeled with (H) an oligodendrocyte-specific antibody to CNPase and (I) BrdU. The arrowhead in (H) indicates a cytoplasmic process extending from the soma. (J) Donor-derived astrocytes (clone H6) in the adult subcortical white matter (indicated by the arrow) and striatum following neonatal intraventricular implantation, immunostained with a human astrocyte-specific anti-GFAP antibody. Inset is higher magnification. (K–Q) Expression of *v-myc* by human NSC clone H6 (K–N) 24 hours and (O–Q) 3 weeks following engraftment in the SVZ. (K,M,O) DAPI nuclear stains of the adjacent panels (L,N,P), immunostained for *v-myc* and (Q) immunostained for BrdU-positive donor-derived cells. (Q) is same as (P). Scale bars: (A and K): 100 μm; (D and E): 10 μm; (O): 50 μm.

Human NSCs integrated into the SVZ within 48 h following implantation (Figs. 4A and B, 5K–N). As with endogenous SVZ progenitors, engrafted human NSCs migrated out along the subcortical white matter by 2 weeks following engraftment (Fig. 4C), and, by 3–5 weeks had appropriately differentiated into oligodendrocytes and astrocytes (Fig. 5A and H–J). The ready detection of donor-derived astrocytes in vivo (Fig. 5J) contrasts with the initial absence of mature astrocytes when human NSC clones were maintained in vitro in isolation from the in vivo environment (Fig. 2D). Signals emanating from other components of the murine CNS appear necessary for promoting astrocyte differentiation and/or maturation from multipotent cells.

Endogenous SVZ progenitors also migrate anteriorly along the RMS and differentiate into OB interneurons[7]. By 1 week following transplantation, a subpopulation of donor-derived human cells from the SVZ migrated along the RMS (Fig. 4D and E). In some cases, these cells migrated together in small groups (Fig. 4E), a

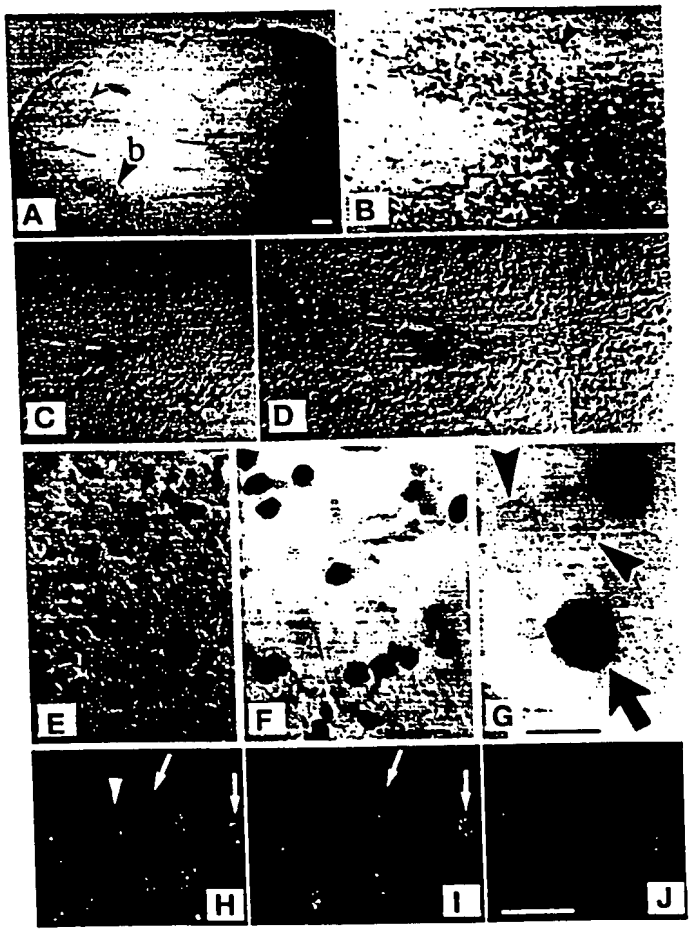

Figure 6. Transplantation of human NSCs into granule neuron-deficient cerebellum. (A–G) Donor-derived cells (clone H6) identified in the mature cerebellum by anti-BrDU immunoperoxidase cytochemistry (brown nuclei) following implantation into and migration from the neonatal *mea* EGL. (A) The internal granule cell layer (IGL and arrowheads) within the parasagittal section of the cerebellum. (B) Higher magnification of the posterior lobe indicated by "b" in (A). (C–G) Increasing magnifications of donor-derived cells within the IGL of a *mea* anterior lobe (different animal from [A,B]). (G) Normarski optics: residual host granule neurons indicated by arrowheads, representative BrDU positive donor-derived neuron indicated by the arrow. (H) Colabeling with anti-BrDU (green) and (I) NeuN (red) indicated with arrows. Arrowhead indicates BrDU+/NeuN- cell. (J) Fluorescent in situ hybridization of cells within the IGL using a human-specific probe (red). Scale bars: (A and B): 100 μm; (F, G, and J): 10 μm.

Table 1. Human neural stem cell clones.

Clone	Propagation technique	Engraftable
H1	bFGF	+
H6	v-*myc*	+
H9	v-*myc*	+
E11	v-*myc*	+
D10	v-*myc*	–
C2	v-*myc*	–

behavior typical of endogenous murine SVZ precursors[31]. Three weeks following transplantation, a subpopulation of donor-derived neurons (human-specific NF-positive cells) were present within the parenchyma of the OB, intermingled with host neurons (Fig. 5B–G). Not only were these donor-derived cells human NF-positive (Fig. 5D), but, when sections through the OB were reacted with both an antibody against BrDU (to identify prelabeled donor-derived human cells) and with an antibody to the mature neuronal marker NeuN, a large number of double-labeled BrDU+/NeuN+ donor-derived cells were integrated within the granule layer (Fig. 5E–G), mimicking the NeuN expression pattern of endogenous, host, murine interneurons (Fig. 5F and G).

Identical clones were implanted into a different germinal zone at the opposite end of the neuraxis to determine their plasiticity. Transplants of the same human NSCs into the EGLs of newborn mouse cerebella appropriately yielded different neuronal cell types in this different location, primarily cerebellar granule cells in the IGL (Fig. 6A–I), detailed below.

Therefore, in vivo—as in vitro (Fig. 2)—all engraftable human NSC clones gave rise to cells in all three fundamental neural lineages: neurons (Figs. 5D–G and 6), oligodendrocytes (Fig. 5H and I), and astrocytes (Fig. 5J). Not only did transplanted brains look histologically normal (donor cells migrated and integrated seamlessly into host parenchyma yielding no discernible graft margins), but engrafted animals exhibited no indications of neurologic dysfunction. Thus, structures that received contributions from donor human NSCs appeared to have developed normally.

Although most clones engrafted well, two appeared to engraft poorly (Table 1). Nevertheless, in vitro these clones displayed characteristics seemingly identical to those of the more robustly engrafting clones. Thus, ostensibly equivalent multipotency in vitro does not necessarily translate into equivalent potential in vivo, suggesting that each clone should be individually tested. This observation also suggests that transplantation of mixed polyclonal populations, because of their shifting representations of various clones, may be a problematic strategy.

Foreign transgene expression in vivo. Many CNS gene therapy needs require that donor cells express foreign genes in widely disseminated locations[32] (in addition to being able to do so in anatomically restricted regions[33]). Murine NSC clones have this capacity[31,34]. Human NSCs appear similarly capable. A representative retrovirally transduced, *lacZ*-expressing clone (Fig. 5A–C) continued to produce β–galactosidase after migration to, and stable integration and maturation within, host parenchyma at distant sites in the mature animal.

Spontaneous constitutive downregulation of *v-myc* expression. In the case of genetically manipulated human NSC clones, the propagating gene product *v-myc* is undetectable in donor human cells beyond 24–48 h following engraftment (Fig. 5K–Q) despite the fact that the brains of transplant recipients contain numerous stably engrafted, healthy, well-differentiated, nondisruptive, donor-derived cells (Figs. 4, 5A–J and Q, and 6). Identical findings have been observed with *v-myc*–propagated murine NSC clones[31] in which *v-myc* downregulation occurs constitutively and spontaneously and correlates with the typical quiescence of engrafted cells within 24–48 h posttransplantation. These observations suggest that *v-myc* is regulated by the normal developmental mechanisms that downregulate endogenous cellular *myc* in CNS precursors during mitotic arrest and/or differentiation. The loss of *v-myc* expression from stably engrafted NSCs following transplantation is consistent with the invariant absence of brain tumors derived from implanted *v-myc*–propagated NSCs, even after several years in mice[3,10]. As with mouse NSCs, neoplasms are never seen using human NSCs.

Neural cell replacement in vivo. Neurologic mouse mutants have provided ideal models for testing specific neural cell replacement strategies. The *meander tail* (*mea*) mutant is one such model of neurodegeneration and impaired development. *Mea* is characterized by a cell-autonomous failure of granule neurons to develop and/or survive in the cerebellum, especially in the anterior lobe[35]. Murine NSCs are capable of reconstituting the granule neuron-deficient IGL[34]. To assess whether human cells may be comparably effective in replacing neurons in CNS disorders, human NSC clones were engrafted into EGLs of newborn *mea* cerebella. When analyzed at the completion of cerebellar organogenesis, donor-derived human cells were present throughout the IGL (Fig. 6). They possessed the definitive size, morphology, and location of cerebellar granule neurons (Fig. 6E–G), identical to the few residual endogenous murine host granule neurons with which they were intermixed (Fig. 6G). That these replacement neurons were of human origin was confirmed by fluorescence in situ hybridization (FISH), using a human-specific chromosomal probe (Fig. 6J). The neuronal phenotype was confirmed by demonstrating that most engrafted cells in the *mea* IGL were immunoreactive for NeuN (Fig. 6H and I); as in the OB, endogenous interneurons in the IGL similarly express NeuN. Thus, engrafted NSCs of human origin appear sufficiently plastic to respond appropriately to varying local cues for lineage determination; recall that the donor human cells were not initially derived from a postnatal brain or from a cerebellum. Furthermore, human NSCs may be capable of appropriate neural cell replacement, much as murine NSCs are[34,35]. While many gene therapy vehicles depend on relaying new genetic information through established neural circuits—that may, in fact, have degenerated—NSCs may participate in the reconstitution of these pathways.

We have presented evidence that neural cells with stem cell features may be isolated from human brains and emulate NSCs in lower mammals[31], vouchsafing conservation of neurodevelopmental principles and suggesting that this cell type may be applied to a range of research and clinical problems in humans. NSCs may serve as adjuncts to other cellular[36], viral[37], and nonviral[37] vectors, including other human-derived neural cells[38–40]. Not only might the clones described here serve these functions, but our data suggest that investigators may readily utilize NSCs from other human material via a variety of equally safe and effective epigenetic and genetic means. That the methods used here yielded comparable cells suggests that investigators may choose the technique that best serves their needs. Insights from studies of NSCs perpetuated by one strategy may be legitimately joined to those derived from studies using others, providing a more complete picture of NSC biology and its applications.

Experimental protocol

Maintenance and propagation of human NSCs in culture. A suspension of primary dissociated neural cells (5×10^5 cells/ml), initially prepared and stably cultured from the periventricular region of the telencephalon of a 15-week human fetus[41] was plated on uncoated tissue culture dishes (Corning, Cambridge, MA) in the following growth medium: Dulbecco's Modified Eagles Medium (DMEM) + F12 medium (1:1) supplemented with N2 medium (Gibco, Grand Island, NY) to which was added bFGF (10–20 μg/ml) + heparin (5 μg/ml) and/or EGF (10–20 μg/ml). Medium was changed every 5

days. Cell aggregates were dissociated in trypsin-EDTA (0.05%) when >10 cell diameters in size and replated in growth medium at 5 x 10 cells/ml.

Differentiating culture conditions. Dissociated NSCs were plated on poly-L-lysine (PLL)-coated slides (Nunc, Naperville, IL) in DMEM + 10% fetal bovine serum (FBS) and processed weekly for immunocytochemistry (ICC). In most cases, differentiation occurred spontaneously. For astrocytic maturation, clones were cocultured with primary dissociated embryonic CD-1 mouse brain[24].

Retrovirus-mediated gene transfer. Two xenotropic, replication-incompetent retroviral vectors were used to infect human NSCs. A vector encoding *lacZ* was similar to BAG[25,26] except for the PG13 xenotropic envelope. An amphotropic vector encoding *v-myc* was generated using the ecotropic vector described for generating murine NSC clone C17.2 (ref. 20) to infect the GP + envAM12 amphotropic packaging line[27]. No helper virus was produced. Infection of bFGF- and/or EGF-maintained human neural cells with either vector (4×10^5 colony-forming units) was as described[28,29].

Cloning of human NSCs. Cells were dissociated, diluted to 1 cell/15 µl and plated at 15 µl/well of a Terasaki or 96-well dish. Wells with single cells were immediately identified. Single-cell clones were expanded and maintained in bFGF-containing growth medium. Monoclonality was confirmed by identifying a single and identical genomic insertion site by Southern blot analysis for either the *lacZ*- or the *v-myc*–encoding provirus in all progeny as described[29]. The *v-myc* probe was generated by nick translation labeling with ^{32}P dCTP; a probe to the *neo* sequence of the *lacZ*-encoding vector was generated by PCR using ^{32}P dCTP.

Cryopreservation. Trypsinized human cells were resuspended in a freezing solution comprising 10% dimethyl sulfoxide, 50% FBS, and 40% bFGF-containing growth medium and brought slowly to -140°C.

Cross-correction of mutation-induced β-hexosaminidase deficiency. The murine NSC clones C17.2 and C17.2H (ref. 22) were maintained in similar serum-free conditions as the human cells. NSCs were cocultured in a transwell system with primary dissociated neural cultures[30] from the brains of either wild-type or α-subunit null (TSD) neonatal mice[31]. These cultures were prepared under serum-free conditions, plated onto PLL-coated glass coverslips, and maintained in the medium described for NSCs. To assess production of a secretable gene product capable of rescuing the mutant phenotype, NSCs (murine and human) were cultured on one side of a membrane with 0.4 µm pores (sufficient to allow passage of hexosaminidase but not cells). The membrane was immersed in a well at the bottom of which rested the coverslip. After 10 days, coverslips were examined for hexosaminidase activity; for expression of the α-subunit in cells of various CNS lineages; for reduction in GM, storage. Hexosaminidase activity was assayed by standard histochemical techniques using the substrate napthol-AS-BI-*N*-acetyl-β-D-glucuroniside (NASBG)[32]; cells stain increasingly pink-red in direct proportion to their enzyme activity. NASBG staining of dissociated wild-type mouse brain cells served as a positive control for both intensity of normal staining and percentage of NASBG-positive cells (~100%). Neural cell types were identified by ICC with antibodies to standard markers: for neurons, NeuN (1:100; gift of R. Mullen, Chemicorp, Temecula, CA); for astrocytes, GFAP (1:500; Sigma, St. Louis, MO); for oligodendrocytes, CNPase (1:500; Sternberger Monoclonals, Baltimore, MD); and for immature undifferentiated progenitors, nestin (1:1000; Pharmingen, San Diego, CA). The α-subunit of human β-hexosaminidase was detected with a specific antibody[22]. Cells were assessed for dual immunoreactivity to that antibody and to the cell type-specific antibodies to assess which TSD CNS cell types had internalized enzyme from human NSCs. Intracytoplasmic GM, was recognized by a specific antibody[33].

Transplantation. For some models, each lateral ventricle of cryoanesthetized postnatal day 0 (P0) mice was iniected as described[34] with 2 µl of NSCs suspended in phosphate buffered saline (PBS) (4×10^5 cells/µl). For other models, 2 µl of the NSC suspension were implanted into the EGL of each cerebellar hemisphere and the vermis as described[35]. All transplant recipients and untransplanted controls received daily cyclosporin 10 mg/kg given intraperitoneally (Sandoz, East Hanover, NJ) beginning on day of transplant. CD1 and *nca* mouse colonies are maintained in our lab.

Detection and characterization of donor human NSCs in vivo. Brains of transplanted mice were fixed and cryosectioned as described[36] at serial time points: P1, P2, and weekly through 5 weeks of age. Prior to transplantation, some human cells were transduced with *lacZ*. To control for and circumvent the risk of transgene downregulation, cells were also prelabeled either by in vitro exposure to BrDU (20 µM; 48 h prior to transplantation) and/or with the nondiffusible vital fluorescent membrane dye PKH-26 (immediately prior to transplantation as per Sigma protocol). Engrafted cells were then detected, as appropriate, by Xgal histochemistry[36]; by ICC with antibodies against β-galactosidase[37] (1:1000, XXX, Durham, NC), BrDU (1:10; Boehringer, Indianapolis, IN), human-specific NF (1:150; Boehringer), and/or human-specific GFAP (1:200; Sternberger Monoclonals); by FISH using a digoxigenin-labeled probe complementary to regions of the centromere present uniquely and specifically on all human chromosomes (Oncor, Gaithersburg, MD); and/or by PKH-26 fluorescence (through a Texas Red [TR] filter), with nondiffusibility having been verified for NSCs. Cell type identity of donor-derived cells was also established as necessary by dual staining with antibodies to neural cell type-specific markers: anti-NF (1:250; Sternberger) and anti-NeuN (1:20) to identify neurons; anti-CNPase (1:200–1:500) to identify oligodendrocytes; and anti-GFAP (1:150) to identify astrocytes. Immunostaining used standard procedures[38] and a TR-coniugated secondary antibody (1:200; Vector, Burlingame, CA). Immunoreactivity to human-specific antibodies also used standard procedures and a fluorescein-conjugated antimouse IgG secondary antibody (1:200; Vector). To reveal BrDU-intercalated cells, tissue sections were first incubated in 2N HCl (37°C for 30 min), washed twice in 0.1 M sodium borate buffer (pH 8.3), washed thrice in PBS, and permeabilized before exposure to anti-BrDU. Immunoreactivity was revealed with either a fluorescein-conjugated (1:250; Jackson, West Grove, PA) or a biotinylated (1:200; Vector) secondary antibody. *V-myc* expression (unique to donor-derived cells) was assessed with an antibody to the protein (1:1000; UBI, Lake Placid, NY). To visualize cellular nuclei, sections were incubated in the blue fluorescent nuclear label DAPI (10 min at 20°C). FISH for the human-specific centromere probe was performed on cryosections from 4% paraformaldehyde/2% glutaraldehyde-fixed brains that were permeabilized, incubated in 0.2 N HCl, exposed to proteinase K (100 µg/ml in 0.1M Tris, 0.005 M EDTA [pH 8.0]), washed (0.1% glycine), and rinsed (50% formamide/2× SSC). Probe was then added to the sections, which were coverslipped, denatured (100°C for 10 min), hybridized (15 h at 37°C), and washed (per manufacturer's protocol). Probe was detected by an antidigoxigenin TR-conjugated antibody (Boehringer) diluted 1:5 in 0.5% bovine serum albumin + 5% normal human serum in PBS. For some donor cells, multiple detection techniques were performed.

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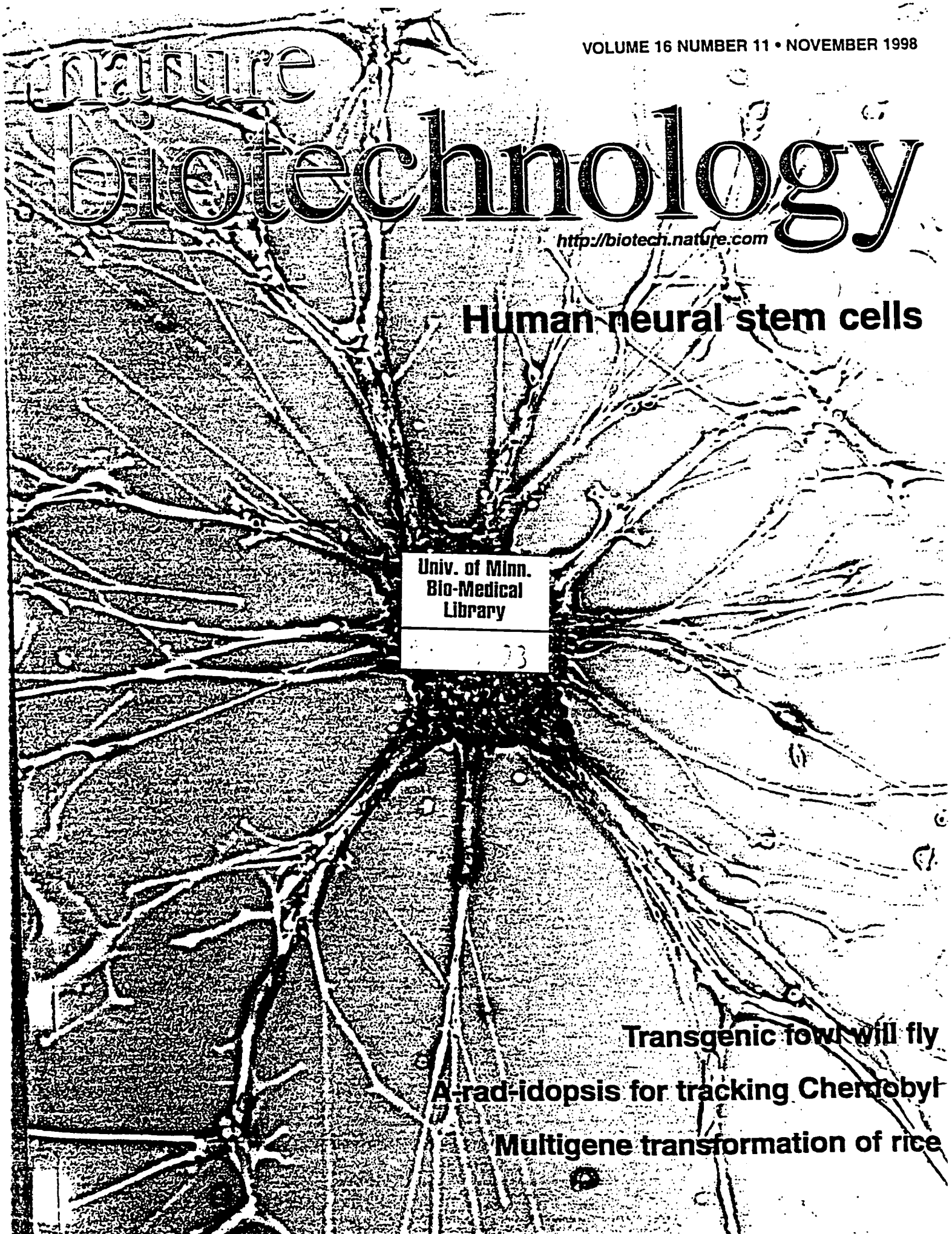

Human neural stem cells

Transgenic fowl will fly

A-rad-idopsis for tracking Chernobyl

Multigene transformation of rice

Chimeric brains generated by intraventricular transplantation of fetal human brain cells into embryonic rats

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Limited experimental access to the central nervous system (CNS) is a key problem in the study of human neural development, disease, and regeneration. We have addressed this problem by generating neural chimeras composed of human and rodent cells. Fetal human brain cells implanted into the cerebral ventricles of embryonic rats incorporate individually into all major compartments of the brain, generating widespread CNS chimerism. The human cells differentiate into neurons, astrocytes, and oligodendrocytes, which populate the host fore-, mid-, and hindbrain. These chimeras provide a unique model to study human neural cell migration and differentiation in a functional nervous system.

Keywords: stem cell, neural progenitor cell, cell therapy

Detailed knowledge of the molecular signals controlling human precursor cell migration and differentiation is a prerequisite for the understanding of human central nervous system (CNS) development. While individual aspects of cell migration and differentiation are accessible in vitro, the molecular interactions governing these events in a complex system such as the developing CNS can be studied only in vivo. Data on neural migration and differentiation in an intact nervous system are particularly important for the design of cell replacement strategies for the treatment of human CNS disorders. An experimental model that permits the analysis of normal and disease-derived human neurons and glia in an unperturbed nervous system would greatly facilitate the study of human CNS development, disease, and repair.

Self-renewing multipotential neural stem cells can be isolated from both the embryonic and adult rodent brain and generate all three major cell types of the CNS[1,2]. Similarly, human neural precursors can be cultured in the presence of basic fibroblast growth factor (FGF2) and, upon growth factor withdrawal, differentiate into neurons, astrocytes, and oligodendrocytes[3,4]. To analyze the properties of human neural precursors in vivo, we have developed a transplant paradigm in which human cells are individually incorporated into a xenogeneic host brain without eliciting traumatic or immunological reactions. Human donor cells were not implanted into the brain tissue but merely deposited in the cerebral ventricles of embryonic rats, allowing them free access to large areas of the neuroepithelium[5–7]. The human donor cells left the ventricle and migrated in large numbers into the rat brain where they differentiated along with the endogenous cells into neurons and glia. We propose that this new approach can be used for the in vivo study of the biological properties of primary and disease-derived human neural precursors as a prelude to the design of therapeutic strategies for neurodegenerative diseases.

Results

Widespread incorporation of transplanted human precursors. Human neural precursors isolated from fetal brain fragments recovered 53–74 days postconception were transplanted immediately or after culture in defined medium containing FGF2 and/or epidermal growth factor (EGF), which promote growth of multipotent rodent neural precursors in vitro[1,2]. Cells were either grown as monolayer cultures or propagated in uncoated tissue culture dishes to form floating spheres[3,10]. In both types of cultures, differentiation into neurons, astrocytes, and oligodendrocytes could be readily induced by growth factor withdrawal[3].

Using intrauterine surgery, human donor cells were grafted into the telencephalic vesicle of embryonic day (E)17–E18 rats[11]. The transplanted cells were traced by DNA in situ hybridization with a human-specific probe to the *alu* repeat element[12] and immunohistochemistry with a human-specific antibody to glutathione-S-transferase (GSTπ). One to eight weeks after transplantation, recipients of acutely dissociated (n=12) and growth factor-treated preparations (n=32) showed incorporated human cells in a variety of gray matter regions, including olfactory bulb, cortex (Fig. 1A), hippocampus, striatum (Fig. 1B and 2), septum (Fig. 3C), tectum (Figs. 1C and 3F), thalamus (Fig. 3D), hypothalamus (Figs. 4D–F), and brain stem. Human cells were symmetrically distributed in recipient brains grafted with single cell suspensions (Fig. 3C). Animals examined during the first postnatal week also exhibited small clusters of residual donor cells attached to the ventricle walls (Fig. 3A). Transplanted spheres were entrapped in periventricular locations and gave rise to a halo of cells that migrated long distances into the host brain (Fig. 3D). Seven to eight weeks after transplantation, sphere-derived cells were found distributed over large areas of the recipient brain (Fig. 1D–E, 3F, 4A–H, 5B). Both freshly dissociated and cultured human neural precursor cells were incorporated into the host white matter. Recipient animals killed between 1 and 7 weeks of age showed abundant GSTπ-positive cells in the major fiber tracts such as the internal capsule (Fig. 1D), corpus callosum (Fig. 1D, inset), anterior and posterior commissures, stria medullaris, fornix, fimbria, as well as fiber tracts in pons and brain stem (Fig. 4G–H). In addition, several recip-

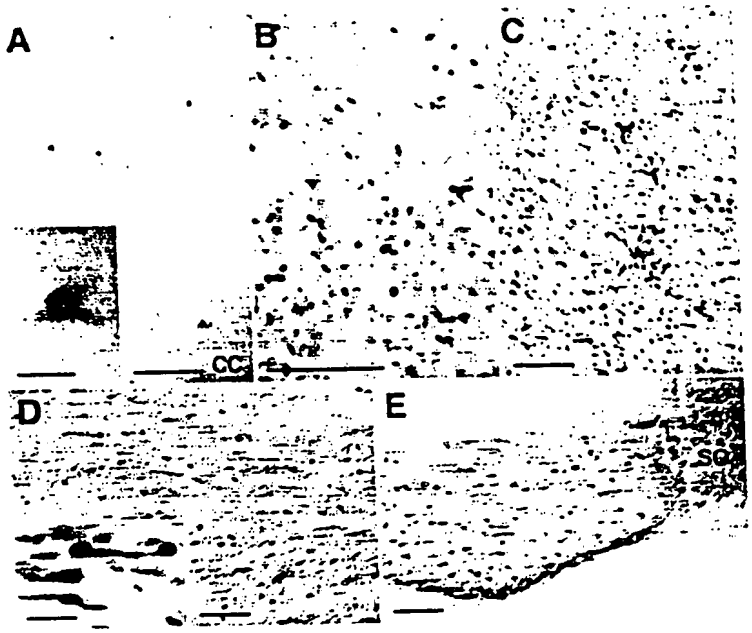

Figure 1. Incorporation of human neural precursor cells into the developing rat brain, visualized by human-specific DNA in situ hybridization (dark nuclear labeling). (A–C) Freshly dissociated cells and (D, E) cells derived from 7-week-old EGF-generated spheres. (A) cortex (postnatal day [P] 30; inset: hybridized nucleus); (B) striatum (P16); (C) inferior colliculus (P16); (D) internal capsule (P45) and corpus callosum (inset); (E) optic nerve (P45). (B), (C), and the inset in (D) are counterstained with hematoxylin to visualize host nuclei. cc: corpus callosum; so: supraoptic nucleus. Bars = 100 μm (insets: 20 μm).

ients exhibited prominent accumulations of human cells in the optic nerve (Fig. 1E). In some animals, the transplanted cells replaced large parts of the subventricular zone (SVZ) of the lateral ventricles. Two months after transplantation, these cells appeared to have migrated from the SVZ into the corpus callosum and adjacent cortical and striatal regions (Fig. 2). Numerous donor-derived cells were also found in white matter and cortex of the cerebellum (Fig. 3E). In some instances, the transplanted cells accumulated around host blood vessels or formed long chain-like structures extending into host gray and white matter (data not shown). Thus, human cells, like rodent cells[9,10,13], can engraft at various levels of the neuraxis following transplantation into the ventricle of embryonic hosts.

In vivo differentiation of human neural precursors. During the first 2 postnatal weeks, human donor cells detected with the GSTπ antibody frequently exhibited uni- or bipolar morphologies characteristic of a migratory phenotype with a leading process and a trailing cell body (Fig. 3B). Human cells incorporated into the molecular layer of the cerebellum maintained immature phenotypes with radially oriented processes for more than 7 weeks (Fig. 3E). At this stage, many of the GSTπ-labeled human cells in other brain regions had acquired multipolar oligodendroglial morphologies (Fig. 3F) and displayed immunoreactivity to an antibody recognizing oligodendrocyte-specific glycolipids (Fig. 4A–C). In addition, these cells expressed myelin basic protein (MBP) in both the cell body and within processes extending to myelin internodes, suggesting active myelination[9] (Figs. 4D–F). These data are compatible with studies showing

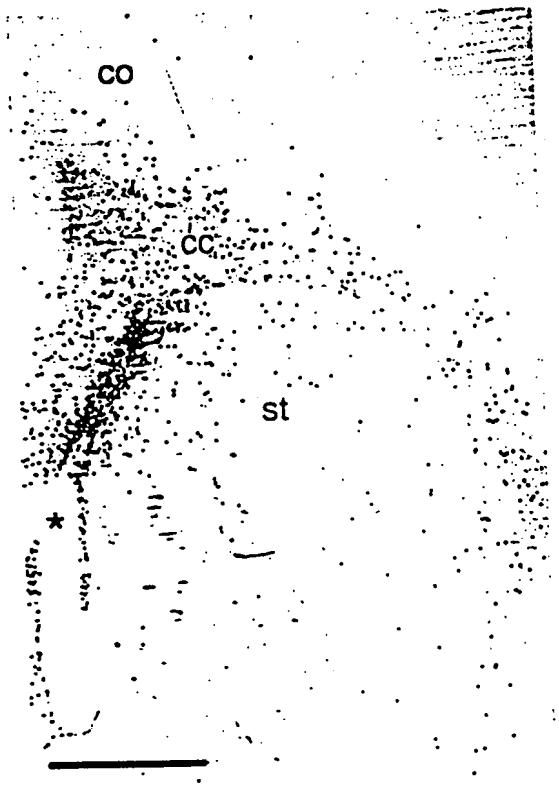

Figure 2. Human neural precursors grown for 6 weeks as monolayers in EGF- and FGF-containing media incorporated into the subventricular zone of the lateral ventricle and migrating into corpus callosum (cc), striatum (st), and cortex (co). Shown is a 50 μm vibratome section through a 7-week-old rat brain. Cells hybridized with the human *alu* probe are labeled with red dots. *lateral ventricle. Bar = 1 mm.

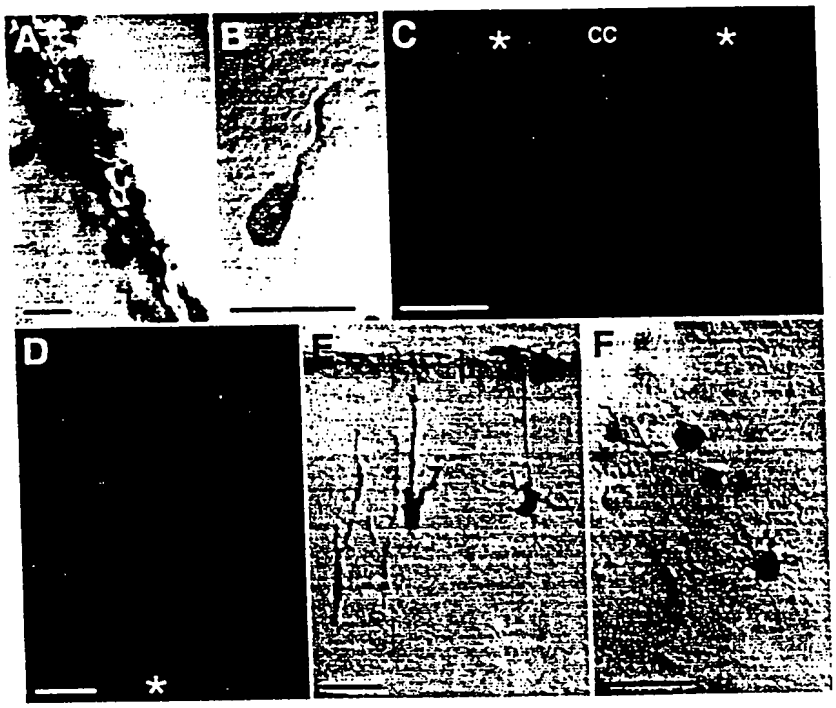

Figure 3. Morphological features of human neural precursors transplanted into the embryonic rat brain, visualized with a human-specific antibody to GSTπ. (A) Residual donor cells attached to the ventricle wall of a 3-day-old recipient animal. (B) Donor cell migrating through the striatal subventricular zone of a neonatal host. (C) Incorporation of freshly dissociated human donor cells in the septum of a 2-week-old host. (D) A human neural sphere grown for 6 weeks in EGF- and FGF-containing media incorporated into the thalamus of a 7-week-old host. (E) GSTπ-positive cells with immature radial phenotypes in the cerebellar molecular layer of a P45 animal. (F) Donor cells with multipolar oligodendroglial morphologies in the tectum, 8 weeks after transplantation of EGF-generated spheres. *ventricles; cc: corpus callosum; p: pial surface. (A, B, E, and F) immunoperoxidase; (C–D) immunofluorescence. Bars = 20 μm (A, B, E, and F), 1 mm (C), 200 μm (D).

strong GSTπ expression in rodent oligodendrocytes[11]. The presence of human oligodendrocytes was confirmed by immunohistochemical detection of the myelin protein 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP) in cells hybridized with the *alu* probe (Figs. 4G and H). Some of the donor cells appeared to form CNP-positive sheaths around host axons (Fig. 4H). Although there are presently no antibodies that would distinguish human from rat CNS myelin, these patterns of MBP and CNP expression suggest that the transplanted human oligodendrocytes myelinate host axons.

Human astrocytes incorporated into the rat brain were identified by double labeling of hybridized cells with an antibody to glial fibrillary acidic protein (GFAP; Fig. 5A and B). These cells were also immunoreactive to an antibody to human adrenoleukodystrophy protein (ALDP), a peroxisomal protein strongly expressed in

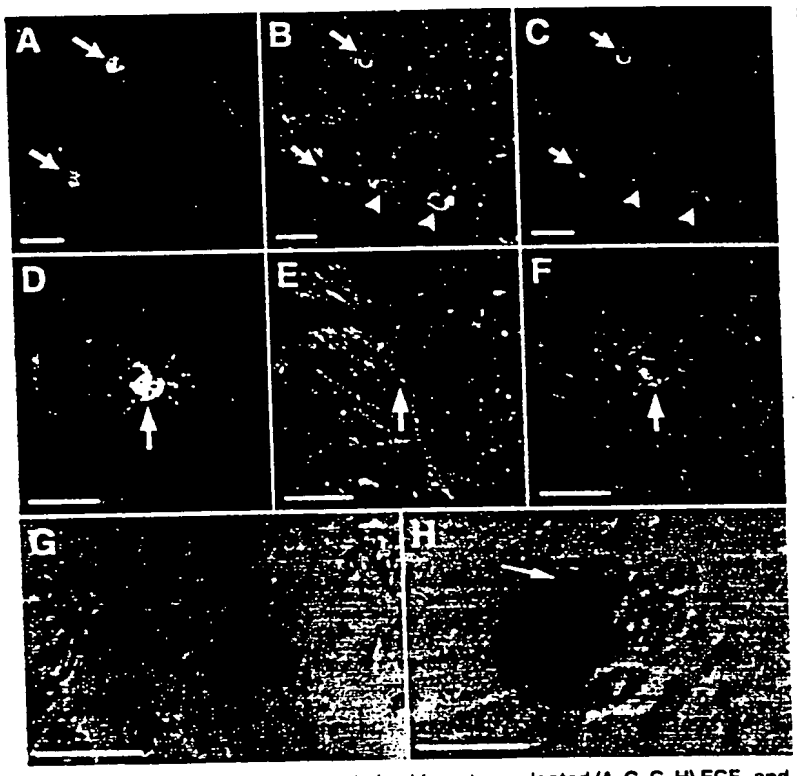

Figure 4. Human oligodendrocytes derived from transplanted (A–C, G–H) EGF- and (D-F) EGF/FGF2-generated spheres incorporated into the brain of 7-week-old rats. (A–C) Donor (arrows) and host (arrowheads) oligodendrocytes in the cortex, double labeled with antibodies to GSTπ (A and C: green) and O4 (B and C: red). (D–F) Human oligodendrocyte incorporated in the host hypothalamus, coexpressing GSTπ (D and F: green) and MBP (E and F: red). (G–H) Human oligodendrocytes in fiber tracts of the ventral brain stem, hybridized with the human *alu* probe (black) and double labeled with an antibody to CNP, which is also staining several myelin internodes (G, brown). Arrow in (H) indicates CNP staining around putative adjacent axons. (A–F) immunofluorescence confocal laser microscopy; (G–H) immunoperoxidase. Bars = 20 μm (A–F), 10 μm (G and H).

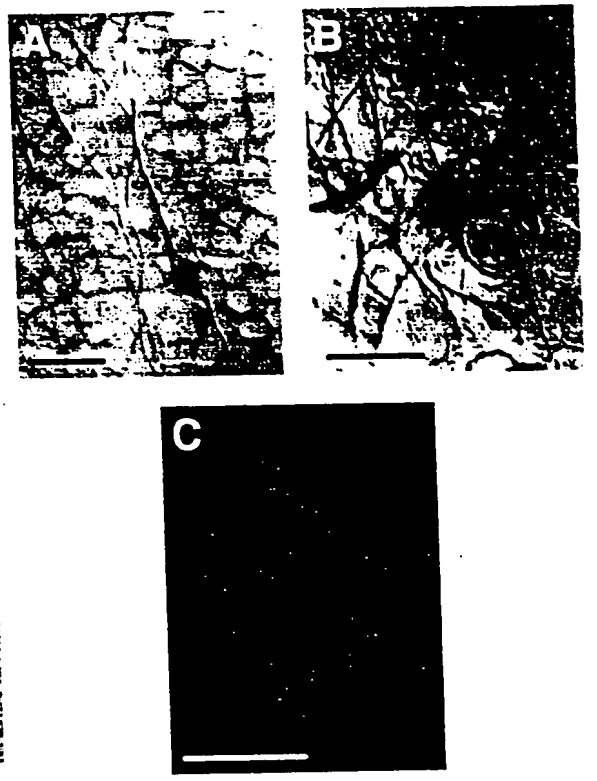

Figure 5. Astrocytic differentiation of the transplanted cells. (A) Hybridized human cell exhibiting radial GFAP-positive processes in the tectum of a 3-day-old recipient. (B) Human astrocyte with a stellate morphology in the tectum of a 7-week-old host, double labeled by in situ hybridization and an antibody to GFAP. Cells are derived from (A) FGF2- and (B) EGF-generated spheres. (C) ALDP expression in a human astrocyte in the ventral telencephalon, 18 days after transplantation of an FGF2-expanded monolayer culture into the ventricle of an E18 rat. Bars = 20 μm.

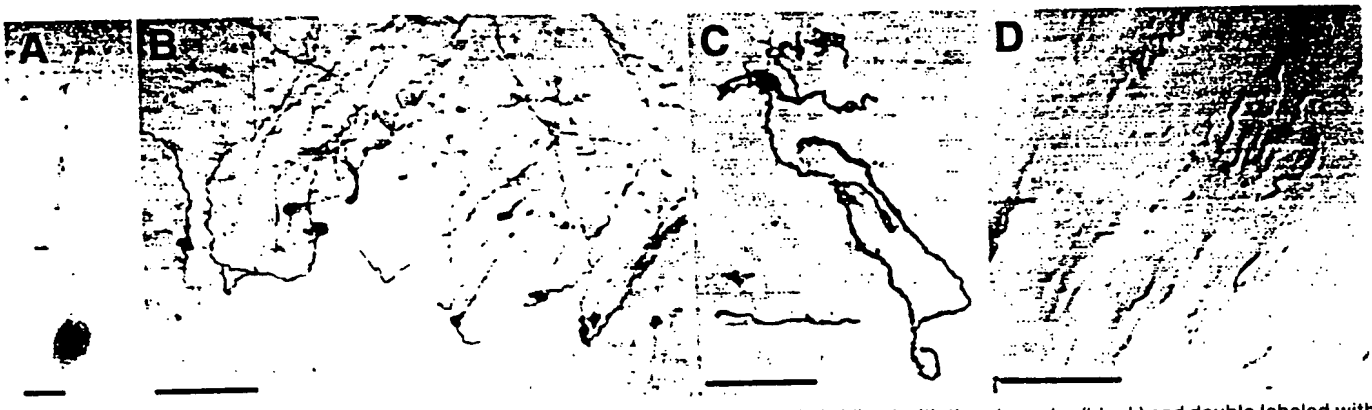

Figure 6. Human neurons incorporated into the rat brain. (A) An individual neuron, hybridized with the *alu* probe (black) and double labeled with a human-specific antibody to neurofilament (hNF-M; brown) in the cortex of a 30-day-old host grafted with freshly dissociated human precursor cells at E17. (B–C) Immunohistochemical detection of β-galactosidase-positive human cells in (B) tectum and (C) hypothalamus of 2-week-old recipients. Donor cells grown for 4 weeks in defined medium containing 10 ng/ml FGF2 were transduced with an adenovirus carrying the *lacZ* gene and transplanted into E17 recipients. (D) hNF-M-positive human axons at the transition of corpus callosum and cortex, 51 days after intraventricular transplantation of 7-week-old EGF-generated neural spheres. Bars = 10 μm (A), 100 μm (B), 50 μm (C), and 20 μm (D).

human astrocytes and microglia[?] (Fig. 5C). Astrocytes with stellate morphologies were found in gray and white matter of forebrain, midbrain, and cerebellum. In neonatal animals, some of the donor-derived astrocytes exhibited conspicuous radially oriented processes (Fig. 5A).

A human-specific antibody to medium-sized neurofilament (hNF-M)[?] was used to visualize transplanted neurons (Fig. 6). Seven to eight weeks after transplantation, numerous immunopositive axonal profiles were detected in the host gray and white matter (Fig. 6D). Donor-derived axons were particularly abundant in cortex and within large fiber tracts such as the corpus callosum and the anterior commissure. As the human hNF-M antibody labeled mostly axons, it only occasionally allowed the identification of neuronal cell bodies (Fig. 6A). In contrast, both neuronal cell bodies and processes could be identified after transplantation of cultured cells infected with an adenovirus harboring the *lacZ* gene, an experiment done to explore the feasibility of gene transfer into transplanted human cells. Immunohistochemical detection of β-galactosidase showed neurons with polar morphologies and long axons incorporated into the host tissue (Figs. 6B and C). β-galactosidase expression was also found in cells with glial morphologies in both gray and white matter, although double immunofluorescent analyses will be required to identify the different types of human cells expressing the transgene.

Discussion

Human neural precursor cells implanted into the cerebral ventricle of embryonic rats incorporate efficiently into the host brain, generating widespread CNS chimerism. Donor cells transplanted as single cell suspensions or spheres migrate into a variety of telencephalic, diencephalic, and mesencephalic regions and differentiate into oligodendrocytes, astrocytes, and neurons. As in mouse-rat neural chimeras[?], no signs of rejection were observed up until at least 2 months after transplantation, indicating immunological tolerance of the transplanted xenogeneic cells by the embryonic rat brain. The ability to incorporate into a xenogeneic recipient brain is maintained after prolonged proliferation of the donor cells in defined, growth factor–containing medium. Moreover, donor cells transduced in vitro with an adenoviral vector continue to express the transgene after incorporation into the host brain.

Previous studies have shown that human neural precursors transplanted directly into the brain tissue of adult immunosuppressed rodents form cell clusters with limited spread of the transplanted cells into the adjacent host brain[?]. In contrast, intraventricular transplantation into embryonic hosts permits widespread delivery of human cells to many brain regions. The widespread distribution of the transplanted human cells is similar to the incorporation pattern observed after intrauterine transplantation of rodent cells[?]. In both cases, the transplanted cells appear to follow endogenous migratory routes. For example, human precursors leaving the ventricle migrated into the optic nerve, where they acquired oligodendroglial morphologies. This observation is reminiscent of the migration of oligodendrocyte precursors from the third ventricle into the optic nerve[?]. Donor cells in the SVZ of the lateral ventricle migrated into the corpus callosum and adjacent cortical and striatal regions (Fig. 2), similar to the migration of glial cells generated postnatally in the rat subventricular zone[?]. These similarities suggest that donor cell migration is not primarily determined by cell-autonomous properties but by guidance cues within the host brain. Responsiveness of human donor cells to migration cues within a rodent brain implies remarkable conservation of these signals across species.

The SVZ serves as an endogenous source of multipotential neural precursors that give rise to neurons and glia throughout life[?]. The ability to introduce human neural precursors into the rat SVZ offers an interesting perspective for the study of cell replacement in the nervous system. Future cell replacement strategies may no longer depend on cell transplantation but focus on the external modulation of endogenous neuro- and gliogenesis by gene transfer and growth factor delivery[?]. Incorporation of human cells into a rodent SVZ provides a unique opportunity to explore the efficacy of these strategies in vivo.

Our data suggest that many of the human cells leaving the SVZ populate the white matter and acquire oligodendroglial phenotypes (Fig. 2). Following migration, differentiated human oligodendrocytes express the myelin proteins MBP and CNP and form CNP-positive sheaths around host axons, suggesting myelin formation (Fig. 4H). Transplants into myelin-deficient mutants will determine the exact amount and distribution of human myelin within the host brain[?]. Thus, the model described might be used and adapted in various ways to study the mechanisms of myelin repair in human demyelinating diseases[?]. As these diseases affect large areas of the CNS, repair of myelin by transplantation would require widespread delivery of oligodendrocytes to the host brain. So far, oligodendrocyte transplants have been generally performed as intraparenchymal grafts, resulting in successful yet spatially restricted remyelination in a variety of animal models[?]. The strategy presented here can be exploited to optimize widespread remyelination in demyelinating diseases. Initially, this approach will be particularly relevant to the question of myelin repair in human leukodystrophies occurring in the perinatal period[?].

Combined with gene transfer into the human donor cells, this chimera model will permit the study of molecular mechanisms regulating human neural migration and differentiation in a functional brain. Alternatively, human donor cells can be introduced into embryos of transgenic mice overexpressing factors known to promote precursor cell migration and differentiation. Such a strategy can be used to assay the effect of trophic factors on human neural cells in a live nervous system. These studies should be particularly useful for the design of cell replacement strategies as well as for probing the efficacy of gene transfer protocols in the human CNS. The lack of traumatic and reactive alterations in the chimeric brains could make this approach a useful tool for the in vivo analysis of neural cells obtained from patients with neurological diseases. Incorporation of affected cells into an unperturbed nervous system may provide new insights into the cellular pathogenesis of these diseases and serve as a model to assay the effects of pharmaceutical agents on human neurons and glia in vivo.

Experimental protocol

Dissociation of human donor cells. Human fetal brain specimens were obtained with consent of the mothers from the Birth Defect Research Laboratory, University of Washington, Seattle (supported by NIH/NICHD grant HD 00836, IRB number 26-0769-A). Cerebral fragments cleaned in sterile conditions and shipped overnight in hibernation buffer[?] typically yielded cell preparations with a viability of 80–95%. Eleven specimens, obtained between 53 and 74 days postconception, were used for this study. Tissue fragments were washed five times in calcium- and magnesium-free Hanks' buffered salt solution (CMF-HBSS) and mechanically triturated to single cell suspensions in the presence of 0.1% trypsin and 0.1% DNAse (Worthington, Freehold, NJ).

Cell culture. Human neural precursor cells were grown in defined medium containing DMEM/F12 (Life Technologies, Rockville, MD), glucose, glutamine, sodium bicarbonate, 25 μg/ml insulin (Intergen, New York), 100 μg/ml human apo-transferrin (Intergen), 20 nM progesterone (Sigma St. Louis, MO), 100 μM putrescine (Sigma), 30 nM sodium selenite (Sigma), penicillin/streptomycin, 10–20 ng/ml FGF2 and/or 20 ng/ml EGF (R&D Systems, Minneapolis, MN). Monolayer cultures were propagated for 1–4 weeks in tissue culture plates coated with fibronectin (Life Technologies; 1 μg/ml) or polyornithin (Sigma; 1.5 μg/ml). Cells were passaged mechanically using a cell scraper. Immediately prior to transplantation, cells were triturated in the presence of 0.1% DNAse. Neural spheres were generated by growing

dissociated cells up to 7 weeks in uncoated tissue culture plates as described. Both types of cultures yielded abundant proliferating cells that expressed nestin, an intermediate filament typically found in neural precursors[1]. Upon growth factor withdrawal, monolayer cultures and plated spheres differentiated into neurons, astrocytes, and oligodendrocytes expressing the cell type-specific antigens β-III tubulin, GFAP, and O4, respectively (A.H. unpublished observations). Growth factor treatment was continued until the day before transplantation. Selected monolayer cultures growing in 10 ng/ml FGF2 were infected with an adenoviral vector carrying the *lacZ* gene under control of the cytomegalovirus immediate-early promoter. Starting 24 h before transplantation, subconfluent 10 cm plates were incubated in 5 ml medium containing 1.5×10^7 pfu of concentrated viral supernatant. Immediately prior to transplantation, cells were washed three times in CMF-HBSS and harvested by incubation in 0.05% trypsin. Following incubation in soybean trypsin inhibitor (Life Technologies), cells were briefly triturated in the presence of 0.1% DNAse.

Intrauterine transplantation. Timed pregnant Sprague-Dawley rats (E17–E18) were anesthetized with ketamine-HCl (80 mg/kg) and xylazine (10 mg/kg), and 0.25–4×10^7 cells (suspended in 1–5 µl CMF-HBSS) were injected into the telencephalic vesicle of each embryo as described[2]. Spheres were sedimented at 150 G for 3 min, washed several times in CMF-HBSS, and implanted using a glass capillary with a 200 µm orifice (20–50 spheres per recipient brain). Larger spheres were mechanically fragmented prior to transplantation. In contrast to recipient animals grafted with mouse cells[3], human-rat neural chimeras showed a high rate of mortality within the first 2 postnatal days (30–40%). Incorporated cells were found in 31 of 44 analyzed recipient brains transplanted with acutely dissociated cells (n = 12; eight positive), monolayer (n = 13; 11 positive), or sphere cultures (n = 19; 12 positive).

Immunohistochemistry. Zero to seven weeks after spontaneous birth, recipients were anesthetized and perfused with 4% paraformaldehyde in phosphate-buffered saline (neonatal animals were fixed by immersion). Serial 50 µm vibratome sections were characterized with antibodies to GFAP (1:100; ICN Biomedicals, Costa Mesa, CA, and 1:500; Sternberger Monoclonals, Baltimore, MD), human glutathione-S-transferase (1:200; Biotrin, Dublin), human ALDP (1:400), phosphorylated medium molecular weight human neurofilament (clone HO14, 1:50), CNP (1:200; Sigma), O4 (1:5; Boehringer Mannheim, Indianapolis, IN), MBP (1:200; Boehringer), and β-galactosidase (1:500; 5Prime3Prime, Boulder, CO). Antigens were visualized using appropriate fluorophore or peroxidase-conjugated secondary antibodies. Specimens were examined on Zeiss Axioplan, Axiovert, and Laser Scan microscopes.

In situ hybridization. Donor cells were identified using a digoxigenin end-labeled oligonucleotide probe to the human *alu* repeat element[4]. DNA-DNA in situ hybridization was performed as described[5]. Briefly, sections were treated with 25 µg/ml pronase in 2× SSC, 5 mM EDTA for 15 min at 37°C, dehydrated, and denatured in 70% formamide, 2× SSC for 12 min at 85°C. After dehydration in cold ethanols, sections were hybridized overnight at 37°C in 65% formamide, 2× SSC, 250 µg/ml salmon sperm DNA. Washes were 50% formamide, 2× SSC (20 min, 37°C), and 0.5×SSC (15 min, 37°C). Hybridized probe was detected using an alkaline phosphatase-conjugated antibody to digoxigenin (Boehringer).

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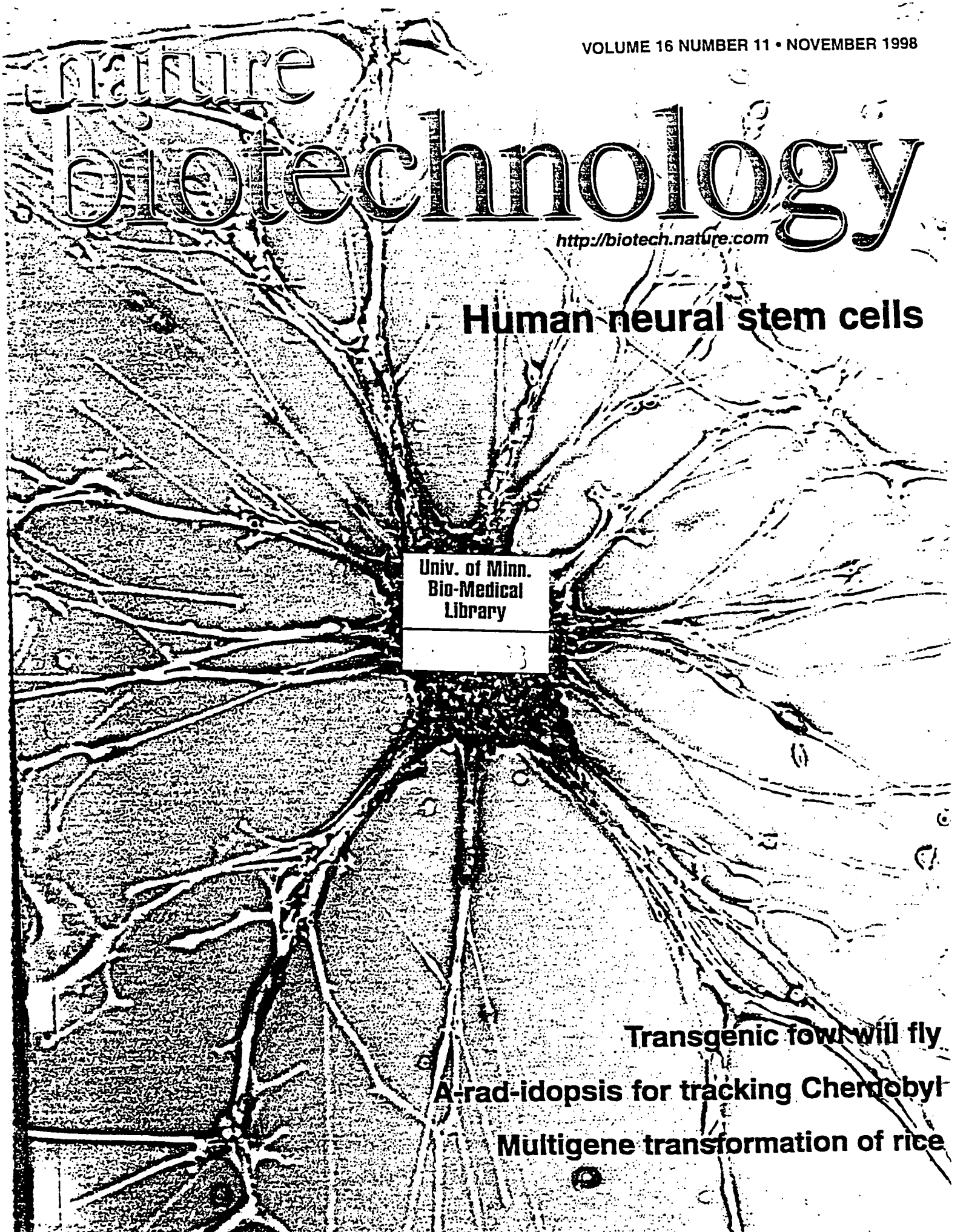

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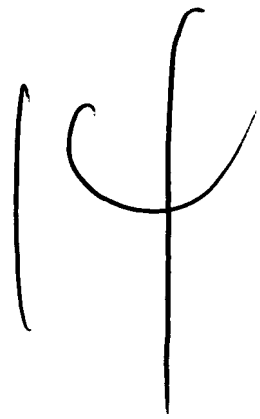

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Adult brain retains the potential to generate oligodendroglial progenitors with extensive myelination capacity

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ABSTRACT **Remyelination of focal areas of the central nervous system (CNS) in animals can be achieved by transplantation of glial cells, yet the source of these cells in humans to similarly treat myelin disorders is limited at present to fetal tissue. Multipotent precursor cells are present in the CNS of adult as well as embryonic and neonatal animals and can differentiate into lineage-restricted progenitors such as oligodendroglial progenitors (OPs). The OPs present in adults have a different phenotype from those seen in earlier life, and their potential role in CNS repair remains unknown. To gain insights into the potential to manipulate the myelinating capacity of these precursor and/or progenitor cells, we generated a homogenous culture of OPs from neural precursor cells isolated from adult rat subependymal tissues. Phenotypic characterization indicated that these OPs resembled neonatal rather than adult OPs and produced robust myelin after transplantation. The ability to generate such cells from the adult brain therefore opens an avenue to explore the potential of these cells for repairing myelin disorders in adulthood.**

Remyelination of the central nervous system (CNS) in patients where host remyelination fails or where the endogenous myelinating cells are genetically impaired may be achieved, at least focally, by glial cell transplantation. It has been assumed that human fetal brain will be the only viable source of myelinating cells for human transplantation, because oligodendroglial progenitors (OPs) derived from embryonic animals have a greater capacity for myelination than mature cells after transplantation (1, 2). However, the availbility of human fetal tissues remains a practical and ethical concern, and it would be preferable if the neonatal or adult human brain could be used as a source of myelinating cells. It has been established that OPs are present in adult human brain (3). Such cells have also been described in patients with multiple sclerosis (4) and in rodents with chronic experimental allergic encephalomyelitis (5). Despite their presence in chronic multiple sclerosis lesions, remyelination may be inadequate (6, 7), and either exogenous myelinating cells must be targeted to lesions or host cells must be recruited to aid in repair.

Two types of OP [also designated *in vitro* as oligodendrocyte type-2 astrocyte (O2A) progenitor] exist in the CNS; the neonatal OP ($O2A^{perinatal}$) that appears in the rat postnatally and disappears about 6 weeks after birth, and the adult OP ($O2A^{adult}$) (8, 9). The $O2A^{adult}$, which is identified by the mAb O4 *in situ* and *in vitro*, has a phenotype that distinguishes it from its neonatal counterpart. The most thoroughly characterized $O2A^{adult}$ cells are those isolated from adult rat optic nerves, although similar cells are found in other parts of the CNS such as the spinal cord (10). Unlike the $O2A^{perinatal}$, the $O2A^{adult}$ does not express the intermediate filament vimentin or a ganglioside recognized by the mAb A2B5. The $O2A^{adult}$ cells also have a longer cell cycle time (65 ± 18 h) and are less motile (4 ± 1 μm/h) than $O2A^{perinatal}$ (9). These characteristics suggest that they would only have a limited capacity to remyelinate demyelinated areas of the brain. In fact, it is not yet known whether these cells produce myelin *in vivo*, for example, after transplantation.

The OPs are generally thought to be derived from multipotent neural precursor cells or early progenitor cells in the CNS. Neural stem cells, which can give rise to both neurons and glia, have been found in the CNS of both embryonic and mature animals (11, 12). Clonal analyses suggest that the stem cells from adult CNS are similar to those of embryonic origin (11). At least, these adult stem cells can differentiate into neurons, astrocytes, and oligodendrocytes *in vitro*. It is not yet known whether adult stem cells differentiate into $O2A^{adult}$ directly.

We have been studying the transition from multipotent precursor cells to lineage-restricted OPs and have shown that it is possible to generate a large number of self-renewing OPs from neural precursor cells derived from embryonic and neonatal brain (13, 14). Because multipotent stem cells exist in adult CNS, we sought to explore whether the OPs derived from adult neural stem or precursor cells have the capacity for extensive myelination. If this were proven in the rodents, a similar approach could provide cells for transplantation or suggest means for the induction of endogenous progenitors to enhance host repair in humans.

MATERIALS AND METHODS

Cell Culture. The neural precursor cells in suspension culture ("neurospheres") were prepared from subependymal striata of Wistar rats aged 3 and 16 months according to a protocol detailed previously (13, 15). The culture medium was DMEM/F-12 (1:1) supplemented with insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM), and sodium selenite (30 nM). The above medium, referred to as "neurosphere medium," was supplemented with 20 ng/ml human recombinant epidermal growth factor (EGF) or EGF plus 20 ng/ml of basic fibroblast growth factor (bFGF) (Collaborative Biomedical Products, Bedford, MA). In the initial week of culture, B27 (GIBCO) was added to the above medium. The cultures were incubated in a humidified atmosphere of 5% CO_2/95% air with a partial medium change every other day.

The B104 neuroblastoma cells were cultured according to Louis *et al.* (16), and the conditioned medium (B104CM) was collected and filtered after 3 days of conditioning the B104 cells with serum-free "neurosphere medium."

BrdUrd Incorporation Assay. The coverslip cultures were incubated in 10 μM BrdUrd (Sigma) for various periods (see

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Abbreviations: CNS, central nervous system; OP, oligodendroglial progenitors; O2A, oligodendrocyte type 2 progenitor; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; MBP, myelin basic protein; GFAP, glial fibrillary acidic protein; PLP, proteolipid; DIV, days *in vitro*.

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Results), fixed in acidic ethanol, and immunostained with anti-BrdUrd antibody (Amersham Pharmacia) at a dilution of 1:10, followed by fluorescein-labeled secondary antibody. For cell cycle time estimation, the cultures were exposed to BrdUrd for a period of 0.5, 1, 3, 5 and up to 16–24 h. The BrdUrd-labeled cells and the total cells stained with Hoechst were counted under a fluorescent microscope. The percentage of the labeled cells was plotted against the time the cells were pulsed, and the cell cycle time was estimated according to the graphic method of Sasaki *et al.* (17).

Assay of Cell Migration. A single sphere was plated onto ornithine-coated 35-mm dishes in a drop of medium. After the sphere attached (10–15 min), 1.5 ml of medium was added gently. Only the samples with successfully attached sphere and without floating cells were followed at 4, 8, and 24 h postplating. The outgrowth of the sphere was examined under the phase-contrast microscope, and the images were photographed and stored in a computer. The longest distance from the edge of a sphere to the cell body in each quarter of the outgrowth was measured and the average distance of cells moved out of a single sphere at specific time points was calculated (13). At least 8 spheres were followed throughout the period of each individual experiment, and the experiment was repeated twice.

Immunocytochemistry. Free-floating spheres or coverslip cultures were immunolabeled with fluorescein-tagged secondary antibodies (Jackson ImmunoResearch) according to the procedure detailed previously (13). The following primary antibodies were used. Monoclonal antibody anti-nestin (IgG) was a supernatant of mouse hybridoma rat401 (diluted 1:5), provided by Developmental Studies Hybridoma Bank (The Johns Hopkins University, Baltimore). A2B5 was a culture medium of mouse hybridoma clone 105 (American Type Culture Collection, CRL-1520, used at 1:100 dilution). O4 and O1 (both were IgM) were provided by M. Schachner. Anti-myelin basic protein (MBP, mouse IgG, 1:100) was from Boehringer Mannheim. Anti-vimentin (mouse IgG) and anti-β_{III}-tubulin (rabbit IgG) were purchased from Sigma (1:100). Polyclonal antibodies anti-glial fibrillary acidic protein (GFAP, 1:200) was purchased from Dako, and anti-platelet-derived growth factor receptor α (PDGFRα, 1:100) was from Santa Cruz Biotechnology.

Transplantation of Oligosphere Cells. The oligospheres were triturated into single cells and were then concentrated to 50,000 cells per microliter. One microliter of cell suspension was transplanted into the spinal cord of postnatal day 6–8 myelin-deficient (*md*) rats according to the procedure described (13, 18). The injection site was marked with sterile charcoal before the incision was sutured.

Twelve to fourteen days after transplantation, the recipient rats were anesthetized with pentobarbital (i.p.) and perfused with 4% formaldehyde. The spinal cord was dissected and the white streak representing myelin made by the transplanted cells was measured. The spinal cords were then trimmed for immunostaining with anti-proteolipid protein (PLP, a gift from I. R. Griffiths, University of Glasgow) or for resin-embedding as described (13, 14).

RESULTS

Establishment of OP Cultures from Adult Rats. The OPs were generated from neural precursors by using the approach described (13, 14). In the present study, cultures of neurospheres were initiated from subependymal striata of adult Wistar rats (aged 3 and 16 months). When cultured in the presence of EGF and absence of substrate, scattered phase-bright cells were found among debris at 4–7 days *in vitro* (DIV). These few cells grew into spheres in the subsequent 2–3 weeks. These spheres were triturated into single cells and expanded in the presence of EGF alone or EGF plus bFGF. Expanded neurosphere cells were immunopositive for nestin (Fig. 1 *a* and *b*), an intermediate filament protein mainly expressed by stem or precursor cells (19). When plated on poly(ornithine)-coated coverslips in the presence of 1% FBS but the absence of EGF or bFGF, the neurosphere cells migrated out and differentiated into a mixture of mainly astroglia (GFAP+) with flattened cell bodies and thick processes and some oligodendroglia (O4+). Some spheres also contained neurons that were β_{III}-tubulin+ (data not shown). The neurospheres were triturated into single cells and passaged in neurosphere medium with the presence of EGF and bFGF. These observations suggest that neurosphere cells are undifferentiated neural precursor cells, similar to those isolated from embryonic and neonatal striatum (13, 14).

To generate OPs from neurospheres, we gradually changed the EGF-containing medium to B104CM-containing medium by replacing one-fourth of the former medium with the latter medium every other day. During the transition period (1–2 weeks), the number and size of spheres did not increase. This is similar to the phenomenon observed in the neurosphere cultures from neonatal rat (13). By week two, the size and number of spheres began to increase. Three to four weeks later, the cultures were passaged in medium containing B104CM (30%) but no EGF or bFGF by plating 1×10^6 cells into a 75-cm^2 flask. New spheres with various sizes formed in

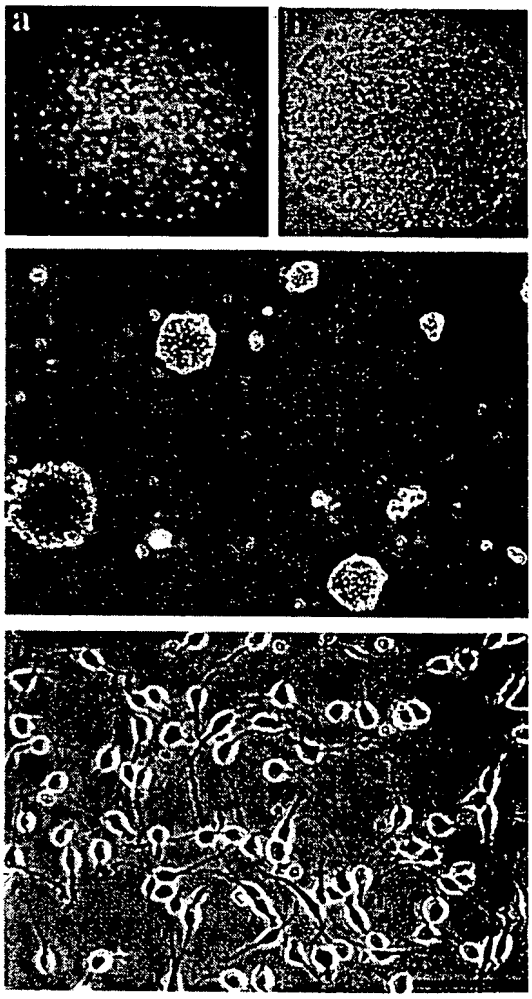

FIG. 1. A neurosphere (from 16-month-old rat) grown in the presence of EGF immunostained with nestin indicated that all cells were nestin+ (*a*). *b* shows the phase-contrast image of *a*. (*c*) New spheres were generated from disaggregated oligosphere cells. (*d*) Disaggregated oligosphere cells displayed bipolar or tripolar morphology in the presence of B104CM. (Bar = 100 μm.)

1 week (Fig. 1*c*). When the spheres were triturated into single cells and plated onto ornithine-coated coverslips, all cells displayed bipolar or tripolar morphology, typical of O2A progenitors (Fig. 1*d*). Therefore, the spheres were now referred to as "oligospheres," a term that was first used by Evercooren and colleagues (24). Similar results were obtained when generating oligospheres from neurospheres that were derived from both 3-month- and 16-month-old rat brains by using the same protocol.

Antigenic Expression of Oligosphere Cells. The $O2A^{perinatal}$ displays a bipolar morphology and is positive for A2B5, whereas the $O2A^{adult}$ is unipolar and O4+ (9). In contrast to the $O2A^{adult}$ previously derived from the adult optic nerve, all oligosphere cells exhibited bi- or tripolar morphology and expressed vimentin, A2B5, and PDGFRα (Fig. 2 *a–c*) when the oligospheres were disaggregated and cultured on ornithine-coated coverslips at a density of 1×10^5 per coverslip in the presence of B104CM. These cells were negative for O4 (Fig. 2*d*). Within a week, the cultures were confluent. Similar results were obtained when the cells were cultured in the presence of both PDGF (10 ng/ml) and bFGF (20 ng/ml) except that they did not reach confluency until about 10 DIV. When the cells were cultured in the presence of PDGF alone with addition of PDGF every other day for 7 DIV, many cells were still bipolar or tripolar (Fig. 2*e*) and the majority were positive for A2B5 ($90.9 \pm 2.4\%$; $n = 5$), vimentin, and PDGFRα. A small number of cells ($5.2 \pm 3.0\%$; $n = 5$), however, became multiprocess-bearing and O4+. In addition, some cells were round without processes. These round cells were positive for A2B5 and vimentin but negative for O4, similar to those seen in the presence of B104CM.

Differentiation of Oligosphere Cells. The $O2A^{adult}$ cells differentiate more slowly than their neonatal counterparts (9). To assess the potential and speed of differentiation, oligosphere cells were cultured in the medium consisting of DMEM and 0.5% FBS. The cultures were immunostained with O4, O1,

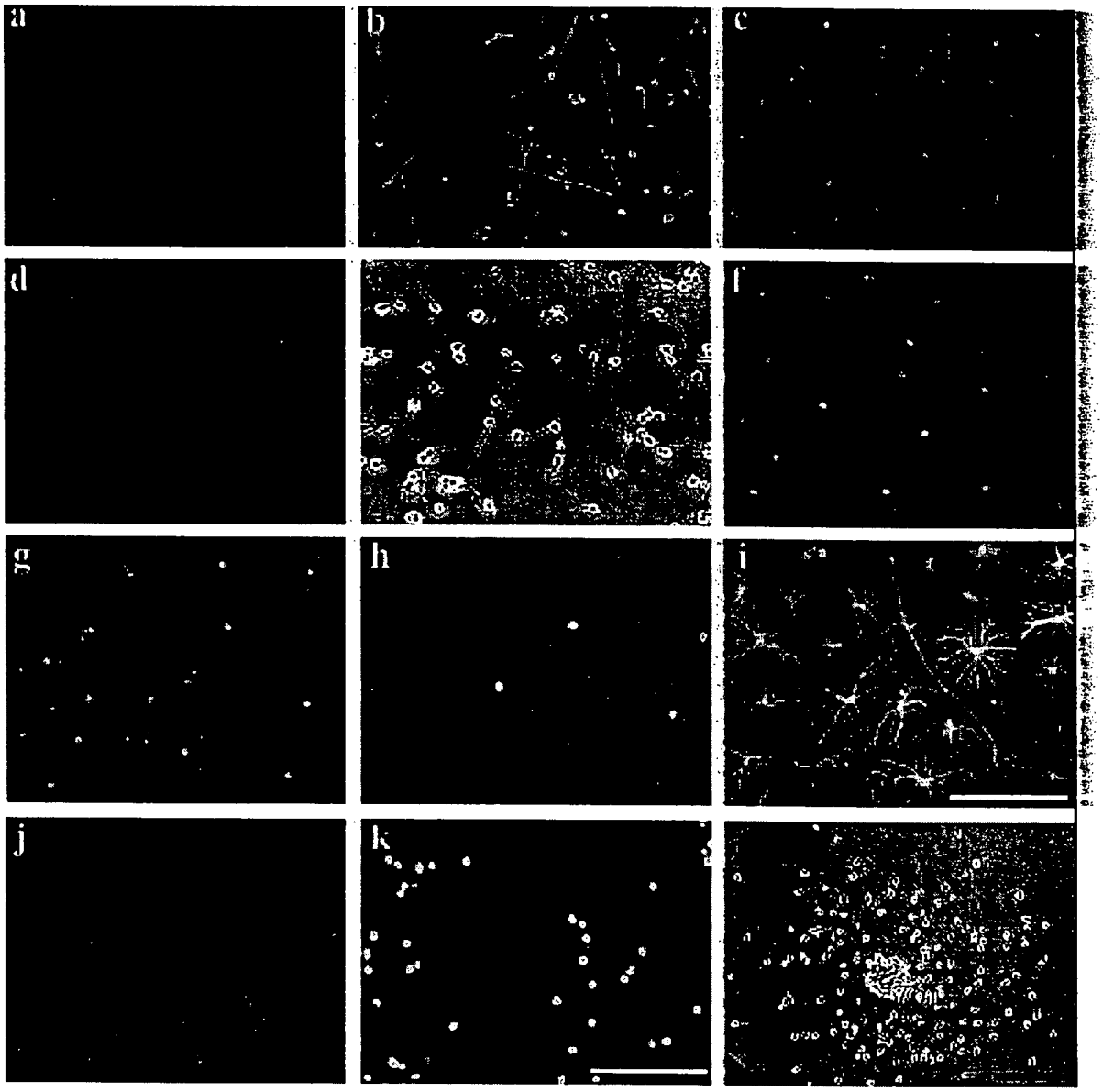

FIG. 2. The oligosphere cells cultured on ornithine-coated coverslips in the presence of B104CM were positive for vimentin (*a*), A2B5 (*b*), and PDGFRα (*c*) but negative for O4 (*d*). In the presence of PDGF alone for 7 DIV, the oligosphere cells were largely bipolar or tripolar. There were also round cells (arrowheads) and a few multiprocess-bearing cells (arrows) (*e*). In the presence of 0.5% FBS for 2 DIV, all cells were O4+ (*f*) and many cells were O1+ (*g*). At 7 DIV, cells were MBP+ (*h*). In the presence of 10% FBS, almost all cells were positive for GFAP (*i*) and A2B5+ (*j*). Incubation of the culture with BrdUrd for 20 h indicated that the majority of cells were labeled in the nuclei (yellow in *k*). All cells were A2B5+ (red in *k*). (*l*) A single sphere plated on ornithine-coated dish in the presence of B104CM for 24 h shows that bipolar cells migrated out of the sphere. The nuclei of cells in (*f–h*) were stained with 4′,6-diamidino-2-phenylindole (DAPI). (Bar = 100 μm.)

and anti-MBP antibodies, which recognize progressively later developmental stages of oligodendroglial lineage. At 2 DIV, virtually all of the cells were O4 + (Fig. 2*f*). At the same time, 57.5 ± 4.4% (n = 6) of the cells were already O1 +, although the staining was mainly in the cell bodies and main processes (Fig. 2*g*). At 3 DIV, the majority of cells were O1 +. At 5–7 DIV, most cells were positive for MBP, displaying membrane-like structures (Fig. 2*h*). In the presence of high concentrations of FBS (5–10%), the majority of cells were flattened, with star-shaped processes, and expressed both GFAP (Fig. 2*i*) and A2B5 (Fig. 2*j*). Similar results were obtained when oligosphere cells of passage 4 or 12 from both ages were examined.

Proliferation Potential. When 1×10^6 oligosphere cells were plated in the presence of 30% B104CM, $(8.8 \pm 1.2) \times 10^6$ (n = 3) cells were obtained in 7 DIV. A similar number of cells were generated when oligospheres from passage 2–12 were examined. The oligospheres could also be expanded in the presence of PDGF plus bFGF, although the yield was lower.

The cell cycle time for oligosphere cells in the presence of B104CM was estimated by using the graphic method described by Sasaki (17). The phase of DNA synthesis was deduced as 6.8–8.4 h from the linear regression of BrdUrd incorporation over incubation time (based on three independent experiments). The total cell cycle time was estimated to be about 20 hours. Incubation of the cells with BrdUrd for 20 h led to ≈92% of the cells labeled with anti-BrdUrd (Fig. 2*k*).

To assess the proliferation potential of oligosphere cells in response to growth factors, oligospheres (passage 4 and 10) were triturated and cultured for 3 days on coated coverslips in the presence of B104CM (30% vol/vol), bFGF (20 ng/ml), PDGF (10 ng/ml), and PDGF plus bFGF. The cultures were then exposed to BrdUrd for 4 h, and the incorporation of BrdUrd into nuclei was assessed. Without the presence of B104CM or above growth factors, cells differentiated into oligodendrocytes (O1 +) and did not incorporate BrdUrd. In the presence of B104CM or growth factors, cells incorporated BrdUrd into their nuclei. The highest percentage of cells incorporating BrdUrd were the cells treated with B104CM (46%), followed by bFGF plus PDGF, bFGF, and PDGF (Table 1). This pattern of growth response of oligosphere cells is similar to that of $O2A^{perinatal}$ cells in response to growth factors (20, 21).

To examine whether a single cell can renew itself and regenerate an oligosphere, a single sphere cell was plated in each well of a 96-well plate containing 200 μl of B104CM (30%)-containing neurosphere medium (13). After 7 days, the plates were reexamined, and the wells containing sphere(s) were marked. The percentage of the cells able to generate new sphere(s) was ≈29% (32/111). The clonally expanded cells retained the same potential to differentiate into oligodendroglia or type-2 astroglia *in vitro* (see above). Similar results were obtained when a single cell was plated into ornithine-coated 96-well plate except that the generated cells did not form a sphere (data not shown).

Migration of Oligosphere Cells. After the oligosphere attached, individual cells migrated out of the sphere within 1 h. At 4 h post-plating, cells were found surrounding the whole sphere. The migration velocity was calculated based on the average distance of cells moving away from the sphere at 4, 8, 12, and 24 hours post-plating. Migration velocity was 25 ± 5.4 μm/h (n = 10) in the presence of B104CM and 13.5 ± 1.7 μm/h (n = 8) in the presence of PDGF (10 ng/ml) for oligospheres (passage 8) derived from the 3-month-old rat. Cells migrating out of the sphere were bipolar (Fig. 2*l*). Unlike the oligosphere cells derived from neonatal rat, the pattern of migration was not always radially oriented. Similar results were obtained when spheres from the 16-month-old rat (passage 6) were examined.

Table 1. BrdUrd incorporation by oligosphere cells

	PDGF	bFGF	PDGF/bFGF	B104CM
BrdUrd+, %	29.5 ± 2.84	34.2 ± 2.27	36.9 ± 3.25	46 ± 4.13

BrdUrd+ cells and total cells were counted in four optic fields of each coverslip. Each group consisted of at least four coverslips. Total cell counts in each group were 3,300–3,965. The data were from the experiment with passage 4 cells derived from a 3-month-old rat.

Myelination Potential by Oligosphere Cells. Oligosphere cells of passage 8 from a 16-month-old rat and passage 4 and 12 derived from a 3-month-old rat were transplanted into the spinal cords of 24 *md* rats. Twelve to fourteen days after transplantation, a white streak, of average 4 mm (3.0–6.5 mm) in length, was present in the dorsal column of the spinal cord of the *md* rat, which is otherwise semitranslucent because of the lack of myelin (Fig. 3*a*). A white streak of 3.9 ± 1.25 mm (n = 7) formed by cells of passage 4 and 3.8 ± 1.5 mm (n = 8) formed by cells of passage 12 that were both derived from the 3-month-old rat. When cells (passage 8) from the 16-month-old rat were transplanted, a white streak of 4.2 ± 1.0 mm (n = 9) formed. There was no difference in the degree of longitudinal spread of transplanted cells and myelination by cells from both ages or cells from passage 4 and 12. A cross section of the spinal cord indicated that the white patch occupied most of the dorsal funiculus. Immunostaining of the spinal cord sections indicated that the myelin sheaths formed by the transplanted cells were positive for PLP (Fig. 3*b*) as well as for MBP (data not shown). The host spinal cord lacks PLP-positive myelin because of a mutation in the PLP gene (22), although PLP + oligodendrocytes were detected in freshly prepared tissues (Fig. 3*b*). Toluidine blue-stained semithin sections (1 μm) confirmed that the majority of axons in the dorsal funiculus were myelinated (Fig. 3*c*). There was no obvious difference between the samples with cells from different ages in terms of the amount of myelin that are present in the transverse section.

DISCUSSION

The major finding of this study is that the adult brain can be used as a source of OPs with the $O2A^{perinatal}$ phenotype and that these cells can be propagated extensively to generate a large number of progenies that maintain their myelinating potential. If similar approaches were feasible in humans, it would be possible to generate large numbers of cells by *ex vivo* manipulation with growth factors, before transplantation. Similarly, it raises the possibility that such cells might be induced to expand by *in vivo* growth factor application and be recruited to target areas of demyelination in the human brain.

Oligosphere Cells Derived from Adult Brain Resemble $O2A^{perinatal}$ Cells. $O2A^{perinatal}$ cells can be isolated and expanded from neonatal rodents by using growth factors or conditioned media when the cells are in peak proliferation (23–25). We have explored alternative means of deriving such cells from multipotential neural precursor cells isolated from neonatal (13) or embryonic rat brains (S.-C.Z., unpublished data) by analogy to the hematopoietic cell lineage development (26). Because multipotential precursor cells exist in the CNS of adult (11, 15) as well as in embryonic stage (12), it is possible that OPs may be generated from adult CNS precursor cells as well. Therefore, the establishment of a homogeneous population of OPs from adult neural precursors was not unexpected. However, that all of the cells were positive for vimentin and A2B5 but negative for O4 contrasts with the antigenic phenotype of the $O2A^{adult}$ as isolated directly from adult rat optic nerves (9). More importantly, the oligosphere cells proliferate much more vigorously and differentiate and migrate faster than the $O2A^{adult}$ progenitors detailed in a series of studies performed by Noble and colleagues (9, 28–30). Therefore, the OPs from adult neural precursor cells resemble neonatal rather than adult O2As isolated directly

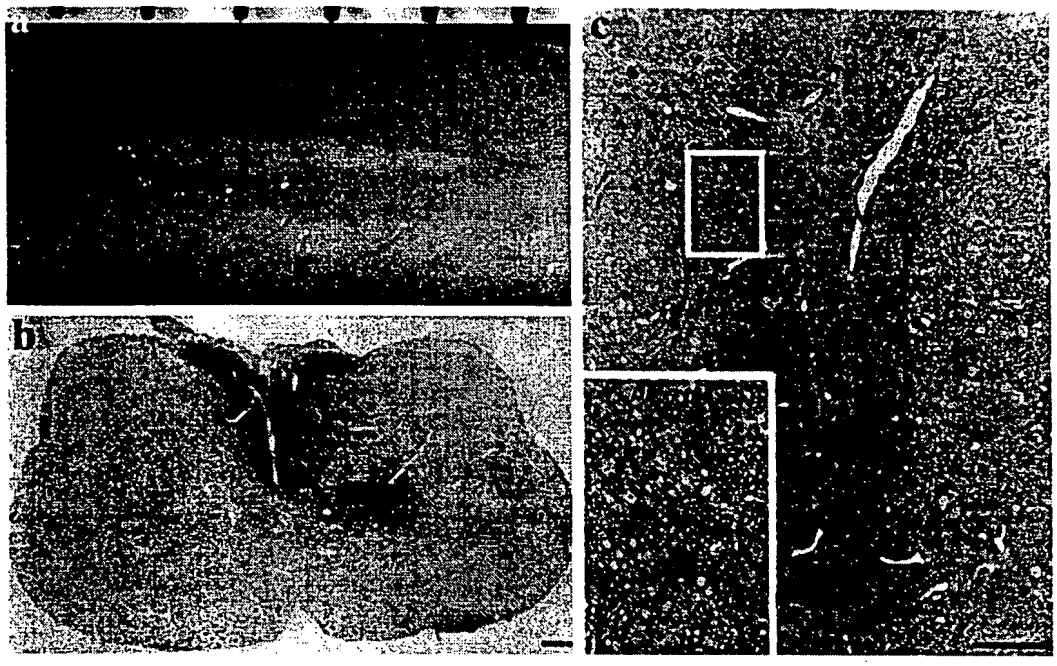

FIG. 3. Transplantation of oligosphere cells from a 16-month-old rat into *md* rats. Twelve to fourteen days later, a white streak of myelin was seen along the dorsal surface of the cord (*a*). The black dots are sterile charcoal marking the injection site. The space bar on top represents 1 mm. (*b*) Immunostaining of the transplanted cord showed PLP+ myelin in the dorsal funiculus with some myelin also appeared in the gray matter. Other areas of the spinal cord showed no PLP+ myelin except the PLP+ cell bodies. (*c*) Semithin sections stained with toluidine blue demonstrated that the dorsal funiculus was occupied by a large number of myelin sheaths. *Inset* is the enlargement of the boxed area in *c*. (Bar = 100 μm.)

from rat optic nerves. This conclusion is further supported by the antigenic expression and proliferation potential of oligosphere cells when they were cultured in the presence of PDGF instead of B104CM, a culture condition similar to that under which $O2A^{adult}$ cells were characterized (9, 27). It should be noted that the population expansion does not parallel the cell cycle time of oligosphere cells. This is mainly due to cell death after mechanical disaggregation and death within spheres. The slower migration in the presence of PDGF alone is potentially accounted for by the techniques used and the growth factors present. Small *et al.* (27) measured the distance a cell moved (in all directions) directly by time-lapse cinematography. We could only measured the linear distance away from the sphere. In the presence of B104CM, the adult oligosphere cells migrated in a similar velocity as neonatal oligosphere cells (13). This result suggests that adult oligosphere cells are similarly motile to neonatal oligosphere cells and that factors other than PDGF also contribute to the migration of OPs. This is further supported by the similar extent of myelination by transplanted adult oligosphere cells as by neonatal oligosphere cells (13) or by the CG4 oligodendroglial progenitor cell line (18).

Oligosphere Cells Are Derived from Neural Precursor Cells. The $O2A^{adult}$ are derived from their neonatal counterparts (29, 31) and may regain the neonatal phenotype temporarily under certain circumstances, such as in the presence of both bFGF and PDGF (30, 32). Is the generation of neonatal-type OPs in the present study attributable to B104CM converting the adult OPs into neonatal progenitors? Our finding does not support this possibility, because the source cells (neurospheres) are nestin+ and the replacement of B104CM with PDGF in oligosphere cell cultures does not lead to the expression of the $O2A^{adult}$ phenotype. We have attempted to generate oligospheres directly from (mechanically and enzymetically) dissociated adult (5-month-old) rat brain and optic nerves by using B104CM. The resultant culture contained floating cells that survived for up to 2 weeks in suspension but did not proliferate (data not shown). A recent observation also indicated that B104CM did not enhance the proliferation of purified $O2A^{adult}$ progenitors (31) or convert the $O2A^{adult}$ to $O2A^{perinatal}$ (B. A. Barres, personal communication). B104CM is a potent mixture in selecting and propagating $O2A^{perinatal}$ in culture (23–25). It may be speculated that some $O2A^{perinatal}$ are selectively expanded by B104CM in the present study. The presence of $O2A^{perinatal}$ in the adult CNS was reported based on their bipolar morphology and A2B5 positivity in a mixed culture (33). However, when the mixed glial cultures were irradiated, no $O2A^{perinatal}$ developed (34), implying that in that study, $O2A^{perinatal}$ cells were being generated *de novo* from A2B5-negative preprogenitor cells that were also present in the cultures. In a purified culture system, the O2A cells from adult (2-month-old) rat optic nerve displayed bipolar morphology and were immunoreactive to A2B5 (31), similar to those reported by Ffrench-Constant and Raff (33). Yet they had a very slow turnover rate (cell cycle time around 3 days), characteristic of $O2A^{adult}$ cells. In our preparation of neurosphere cultures, these rare $O2A^{perinatal}$ (if they are present) would be unlikely to survive in the condition without substrate and survival factors such as PDGF for a long time (>4 weeks). Our previous study (13) indicated that EGF is not a survival factor for OPs in suspension cultures. Our failure to generate oligospheres directly from dissociated adult brain and optic nerves suggests that either there are no $O2A^{perinatal}$ present or such cells do not survive the procedure and culture condition. Therefore, the cells used for generating OPs are unlikely to contain cells that are already in the oligodendroglial lineage. Thus, the present study extends our previous argument that factors in B104CM may induce neural precursor cells to commit to oligodendroglial lineage while at the same time maintain the OPs in a state of self-renewal (13).

Multipotent Neural Precursor Cells as a Source for Remyelination. The generation and extensive propagation of the neonatal type of OPs from the adult rat brain has an important impact on the design of strategies for promoting remyelination *in vivo*. In the first instance, as we show here, it may be possible to similarly derive progenitor cells of the neonatal phenotype from the adult human brain for transplantation. Extensive animal studies suggest that transplantation of myelinating cells, especially their progenitor cells, may be an effective

approach (1, 2, 36, 37). In clinical human trials, however, cell availability becomes a problem if the cells are to be obtained from a source other than the patient. At present, human fetal tissues are the only source of immature neural cells. However, there are long-term practical and ethical concerns on the availability of such tissue, including stringent safety concerns. Here we show that it is possible to generate a large number of OPs from a small source of tissue in the rodent brain. A similar approach may be possible by biopsy from the human brain with *ex vivo* conversion of neural precursors to OPs with subsequent expansion. Such transplantation would therefore be autologous and obviate the need for immunosuppression.

The alternative approach is to recruit endogenous OPs to instigate repair. Cells that are responsible for remyelination in adults are mainly dividing "progenitor cells" (38, 39). The O4+ multiprocess-bearing cells that are regarded as the $O2A^{adult}$ *in vivo* have been found in the CNS of normal and (myelin) diseased animals and humans (4, 5, 40). The apparent lack or limit of remyelination in terms of the universal existence of O4+ $O2A^{adult}$ suggests that either the environment or the cells' intrinsic properties (or both) is responsible. In the presence of (lysolecithin-indueced) demyelination, retrovirus-labeled proliferating progenitors failed to migrate even a short distance (<500 μm) over a period of 4 weeks to perform remyelination (38). Such a poor migration behavior may be intrinsic to the multiprocess-bearing $O2A^{adult}$ rather than due to the nonpermissive environment, because transplanted neonatal OPs migrate a long distance and myelinate axons in dysmyelinated adult CNS (1, 36). Neuronal progenitors can also migrate a long distance from subependymal area to olfactory bulb in adult environment (41). In a separate study by Keirstead *et al.* (39), $O2A^{adult}$ (identified by NG2 labeling) adjacent to focally demyelinated lesions decreased in number with time and were not mitotic, and they suggested therefore that the $O2A^{adult}$ are inherently incapable of regeneration (39). Therefore, strategies designed to simply increase the number of $O2A^{adult}$, such as by delivering PDGF into the CNS (35), may not be effective. An alternative avenue to this strategy, therefore, is to promote the *in vivo* regeneration of the $O2A^{perinatal}$ from host neural precursors or stem cells, in a similar fashion as suggested by the present study. Such cells are present in subependymal areas of the adult CNS and can differentiate into neurons and glia (11), therefore close to commonly affected areas in multiple sclerosis (6). The motility of $O2A^{perinatal}$ might also indicate their ability to migrate to other parenchymal sites. The key to the application of these strategies in humans will be the identification of growth factors that have the biological effects both *in vitro* and *in vivo* on these precursor cells.

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"Global" cell replacement is feasible via neural stem cell transplantation: Evidence from the dysmyelinated *shiverer* mouse brain

(myelination/mutants/oligodendroglia/regeneration)

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ABSTRACT **Many diseases of the central nervous system (CNS), particularly those of genetic, metabolic, or infectious/inflammatory etiology, are characterized by "global" neural degeneration or dysfunction. Therapy might require widespread neural cell replacement, a challenge not regarded conventionally as amenable to neural transplantation. Mouse mutants characterized by CNS-wide white matter disease provide ideal models for testing the hypothesis that neural stem cell transplantation might compensate for defective neural cell types in neuropathologies requiring cell replacement throughout the brain. The oligodendrocytes of the dysmyelinated *shiverer* (*shi*) mouse are "globally" dysfunctional because they lack myelin basic protein (MBP) essential for effective myelination. Therapy, therefore, requires widespread replacement with MBP-expressing oligodendrocytes. Clonal neural stem cells transplanted at birth—using a simple intracerebroventricular implantation technique—resulted in widespread engraftment throughout the *shi* brain with repletion of MBP. Accordingly, of the many donor cells that differentiated into oligodendroglia—there appeared to be a shift in the fate of these multipotent cells toward an oligodendroglial fate—a subgroup myelinated up to 52% (mean = ≈40%) of host neuronal processes with better compacted myelin of a thickness and periodicity more closely approximating normal. A number of recipient animals evinced decrement in their symptomatic tremor. Therefore, "global" neural cell replacement seems feasible for some CNS pathologies if cells with stem-like features are used.**

Many diseases of the central nervous system (CNS) are characterized not by discrete, focal neuropathology but, rather, by extensive, multifocal, or even "global" neural degeneration or dysfunction. Such conditions may require widespread replacement not only of therapeutic molecules, such as enzymes, but of neural cells, as well. "Global" cellular replacement has been regarded as beyond the capabilities of neural transplantation, which previously has been used in situations in which grafts are placed in single, relatively circumscribed, anatomic locations (e.g., the striatum in Parkinsonism). However, we previously demonstrated that neural stem cells (NSCs) can disseminate therapeutic gene products throughout the CNS (1, 2). We hypothesized that transplantation of NSCs also might work in situations requiring "global" replacement of degenerated or dysfunctional neural cells.

An NSC is an immature, uncommitted cell that exists in the developing and even adult nervous system and gives rise to the array of more specialized cells of the CNS (3–15). It is defined by its ability to self-renew, to differentiate into cells of most (if not all) neuronal and glial lineages, and to populate developing or degenerating CNS regions. The recognition that NSCs, propagated in culture, could be reimplanted into mammalian brain, where they could reintegrate appropriately and stably express foreign genes (1–9), provided hope that their use might make feasible a variety of novel therapeutic strategies. When exogenous NSCs are transplanted into germinal zones, they circumvent the blood–brain barrier, migrate to distant CNS regions, and participate in the normal development of multiple regions throughout the brain and at multiple stages (from fetus to adult), integrating seamlessly within the parenchyma, differentiating appropriately into diverse neuronal and glial cell types. Thus, their use as graft material can be considered almost analogous to hematopoietic stem cell-mediated reconstitution of bone marrow. In one of their earliest uses as a therapeutic tool, NSCs were implanted at birth, using a simple, rapid, intracerebroventricular injection technique. They delivered a missing gene product (β-glucuronidase) throughout the brain of a mouse in which the gene was mutated in all cells (the MPS VII mutant, a model of the neurodegenerative lysosomal storage disease mucopolysaccharidosis type VII), cross-correcting the widespread neuropathology of host neurons and glia by creating virtually chimeric brain regions (1). We hypothesized that a similar method might accomplish "global" replacement of degenerated or dysfunctional neural cells.

Mutant mice characterized by CNS-wide white matter disease because of oligodendrocyte dysfunction provide ideal models for testing this hypothesis. The *shiverer* (*shi*) mouse suffers from extensive dysmyelination because of an autosomal recessive defect that renders its oligodendrocytes dysfunctional in homozygous animals: a deletion of five of seven exons comprising the gene encoding myelin basic protein (MBP) makes the cells incapable of producing this oligodendroglial component that is essential for effective compact myelination (16–20). Severe tremors develop by 2–3 weeks of age. Therapy for this cell-autonomous defect would require extensive replacement with functional MBP-producing oligodendrocytes. (In a sense, replacement of both an abnormal neural cell type and a dysfunctional gene is entailed.) It is known that the *shi* cellular and behavioral phenotypes can be rescued by introducing the wild-type MBP gene into the germ line (17). However, this approach is not applicable to clinical therapies. The *shi* phenotype has been treated in discrete regions by injecting a fragment of primary CNS tissue containing normal, mature, MBP-expressing oligodendrocytes (19) in much the same way as other relatively focal, demyelinated lesions have been addressed by cells from various stages within the oligodendrocyte lineage (21, 22). However, this does not correct the global abnormality of *shi* CNS myelin. The most differentiated oligodendrocytes migrate minimally from the injection site, whereas cells that migrate more broadly are less

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Abbreviations: CNS, central nervous system; NSC, neural stem cell; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; β-gal, β-galactosidase; LM, light microscopy; EM, electron microscopy.

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likely to differentiate into mature myelinating cells (23). In contrast to resident oligodendrocytes and more differentiated precursors, multipotent, migratory NSCs tend not to differentiate until instructed by regional cues. This property might circumvent these problems. Therefore, NSCs were transplanted at birth into the brains of *shi* mutants, using the same intracerebroventricular implantation technique devised for diffuse engraftment of enzyme-expressing NSCs to treat global metabolic lesions (1, 2).

MATERIALS AND METHODS

Animals. *Shi* breeders were obtained initially from The Jackson Laboratory, and a colony was maintained thereafter in our facility. With careful husbandry, homozygous *shi* males and females (2–5 months of age) can mate with each other; a *shi/shi* genotype was ensured by using the progeny of such homozygous matings.

Transplantation. On the day of birth, *shi/shi* pups (and unaffected controls) received bilateral intracerebroventricular injections of a suspension of NSCs (clone C17.2) as described (1, 2). C17.2 is a stable, prototypical NSC clone originally derived from neonatal mouse cerebellum but capable of participating in the development of most other regions upon implantation into germinal zones throughout the brain (1, 2, 6, 24–26). The NSCs differentiate into neurons in regions undergoing neurogenesis or into glia, where gliogenesis is ongoing (6, 24, 25). Therefore, they emulate endogenous NSCs as well as NSC clones propagated by a variety of techniques from other structures (3–15). After 0–2 mitoses in the first 48 hr posttransplant, they become quiescent and intermingle nondisruptively with endogenous progenitors. The total cell number per region (host plus donor) is not increased. The clone constitutively and stably expresses *lacZ* [encoding β-galactosidase (β-gal), detectable by the 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) histochemical reaction]. Undifferentiated NSCs, maintained in culture and prepared for transplantation as detailed elsewhere (1, 2, 24–26), were resuspended in PBS at 4×10^4 cells/μl. The lateral ventricles of cryoanesthetized pups were visualized by transillumination of the head (1, 2); 2 μl of the cellular suspension was expelled gently via a glass micropipette inserted transcutaneously into each ventricle (gaining access to the subventricular zone). Pups were returned to maternal care until weaning. Six homozygous litters of eight offspring each were transplanted.

Analysis of Engrafted Brains. At various intervals between 2 and 8 weeks after transplantation, serial coronal sections of recipient brains were processed with X-gal histochemistry and/or an anti-β-gal antibody (Cappel, 1:1,000) to detect *lacZ*-expressing donor-derived cells as detailed previously (25, 27). The phenotypes of those cells were assessed by light microscopy (LM), immunocytochemistry, and electron microscopy (EM) by using predesignated, standard criteria detailed elsewhere (24–30). Although donor NSCs give rise to a range of neurons and glia *in vivo* [as in our previous reports (1, 2, 24–27)], this study was confined to an assessment of donor-derived oligodendroglia and their potential replacement; therefore, for the purposes of this study, all cells, both donor and host, were classified as either "oligodendrocytes" or "not oligodendrocytes." Because multiple modes of analysis could be performed on the same tissue, multiple parameters could be correlated in the same animal.

Morphologic analysis first was performed at the LM level by using bright-field, differential interference contrast (DIC) and/or immunofluorescence microscopy. Engrafted cells then were assessed by using ultrastructural criteria by EM for the direct visualization and quantitative morphometrics of cell type-specific components, including myelin (24–30). X-gal-processed tissue was prepared for EM as detailed previously (24–26). The X-gal reaction product forms a crystalline blue precipitate that is nondiffusible and electron dense, permitting donor-derived *lacZ*-expressing cells to be identified and distinguished unequivocally from unlabeled endogenous cells at both LM and EM levels (24–30) (see Figs. 4*D* and 5*A*). The precipitate typically is localized to the nuclear membrane, forming a nuclear ring within donor-derived cells (often overlying the nucleus), endoplasmic reticulum (ER), and other cytoplasmic organelles, and it frequently extends into cellular processes. Despite the presence of accepted LM features and immunocytochemistry markers, CNS cell types, and, particularly, functional oligodendrocytes, have been most extensively and reliably characterized ultrastructurally. Established criteria for cell-type assignment [detailed elsewhere (16–30)] were used. We also have validated independently these criteria by correlating ultrastructural, histologic, and antigenic profiles of individual donor-derived and host cells as detailed previously (24–30). Briefly, a cell was scored as an "oligodendrocyte" if it possessed the following ultrastructural criteria: was a small (5- to 8-μm diameter), round or oval cell with a smooth, regular perikaryon and a distinctively dark nucleus and cytoplasm (an appearance created by numerous fine granules); possessed a large, prominent nucleus (occupying more than one-half of the area of the cell) with dense heterochromatin marginated against the nuclear membrane and/or clumped centrally; possessed a thin rim of cytoplasm that, though not voluminous, could appear as a mass at the cellular poles when the nucleus lay eccentrically; and possessed a moderate number of short, round mitochondria and often long, meandering, distended ER (16–24, 28–30). Association with myelinated fibers, if visible in the same plane of section, helped confirm an oligodendroglial phenotype. Cells not meeting these criteria were scored as "nonoligodendrocytes."

Morphometric analysis of myelin on electron micrographs entailed assessment of the degree of compaction, including noting the presence of major dense lines (MDLs) (the oligodendroglial cytoplasmic membrane appositions that constitute the wraps of myelin) and quantifying both periodicity (a measure of interlamellar distance between myelin wraps as represented by the distance between intraperiod lines and/or MDLs) and width of the total myelin wrap. Measurements in engrafted regions of *shi* cerebrum were compared with those in unengrafted areas of the same mouse (internal control) and with those in analogous, homotopic regions of the cerebra of age-matched affected and unaffected, unengrafted control mice. Cell counts and morphometrics were performed under EM on randomly, systematically selected representative fields and EM grids from multiple noncontiguous sections spanning the cerebra of experimental and control mice. Cell-type assignments were confirmed independently by three observers blinded to the experiment.

The presence of MBP *in vivo* classically has been used in grafting studies into *shi* (31) to distinguish normal donor from mutant oligodendrocytes. For immunocytochemistry analysis of brain tissue, 20-μm-thick, 4% paraformaldehyde-fixed cryosections or 10-μm-thick, paraffin-embedded sections were reacted with a polyclonal antibody raised in rabbit against MBP (gift of D. Colman, Mt. Sinai; 1:200) by using standard immunoperoxidase (Vectastain, Vector Laboratories) or immunofluorescence procedures as detailed previously (2, 24, 25, 27). For analysis of cultured cells, a cellular monolayer was processed by using standard immunoperoxidase techniques. NSC-derived cells expressed MBP in culture after prolonged periods *in vitro* (>2 weeks) and/or after supplementation with conditioned medium from primary cultures of dissociated mouse cerebrum, a condition that appears to emulate the stably engrafted state.

Western Blot Analysis. Western blot analysis, using standard techniques and the above-referenced anti-MBP antibody, was performed on myelin-enriched membrane fractions (32) of whole-brain lysates prepared from semiserial coronal sections of transplanted *shi/shi* mice at 4–5 weeks of age, as well as age-matched, untransplanted *shi/shi* and unaffected controls.

Behavioral Assessment. Functional improvement in representative recipient animals was assayed by quantifying the amount of tremor by (*i*) scoring recorded cage behavior and (*ii*) measuring

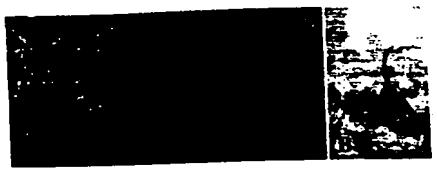

FIG. 1. NSCs can express MBP. Two engraftable NSC clones, known to give rise to oligodendrocytes *in vivo* after transplantation, are reacted with an antibody to MBP *in vitro* by using immunoperoxidase methodology. (*A*) A subpopulation of NSC clone C17.2 (arrows) differentiates into MBP-expressing cells after exposure to conditioned medium from a primary culture of newborn mouse forebrain. (*B*) Cells from clone C27.3 that spontaneously differentiated toward MBP expression. The present experiments were performed by using clone C17.2 because of prior experience with these cells in CNS-wide gene therapy engraftment paradigms.

the amplitude of tail tremor, a readily accessible, reliable, and reproducible measure of whole-body tremor.

Cage behavior of both transplanted (n = 10) and untransplanted (n = 3) affected mice as well as unaffected controls (n = 3) was videotaped and graded independently by three investigators blinded to the experiment. A four-point neurologic scoring scale was used, where a score of "1" corresponded to completely abnormal behavior and a score of "4" denoted a completely normal neurologic exam. Animals were rated according to their (*i*) exploring/grooming behavior, (*ii*) voluntary movement, (*iii*) tail movement and degree of tremor, and (*iv*) proprioception, coordination, and posture. Unaffected control mice, on this blinded assessment, achieved a mean score of 3.91 ± 0.14, which was significantly different from the mean score of 2.09 ± 0.08 received by untransplanted *shi* mutant controls ($P < 0.00001$), suggesting the sensitivity and validity of this assay with minimal interobserver variability. Each experimental animal similarly was graded blindly. At the completion of the study, upon breaking of the animals' identity codes, each score was calculated for a statistically significant difference from unaffected and affected/untreated animals. Mean scores of the groups also were compared.

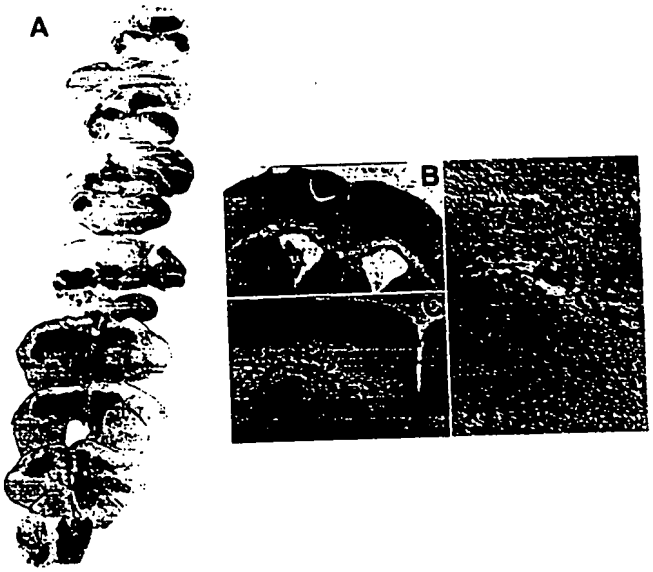

FIG. 2. NSCs engraft extensively throughout the *shi* dysmyelinated brain, including within white tracts. *LacZ*-expressing NSCs were transplanted into newborn *shi* mutants and analyzed systematically at intervals between 2 and 8 weeks after engraftment. (*A*) Semiserial coronal sections through the *shi* brain at adulthood demonstrate widely disseminated integration of blue X-gal$^-$ donor-derived cells throughout the neuraxis. (*B–D*) Progressively higher magnification of donor-derived cell integration in white tracts (arrows) at 2 weeks of age.

Because tremor is the most prominent feature of the *shi* behavioral phenotype, it was quantified directly by measuring the degree of tail displacement perpendicularly from a straight line drawn in the direction of the animal's forward movement (tail amplitude) (see Fig. 6 *C* and *D*). Measurements were made by coating an animal's tail in India ink and then permitting the mouse to move freely in one direction on a sheet of graph paper. The tail of an unaffected nontremulous animal draws a line with virtually no perpendicular displacement from the direction of movement (i.e., the long axis of the body); e.g., amplitude = 0 cm. The tail of a "shivering" animal demarcates a broad region of movement (tremor) about the line (i.e., displacement perpendicular to that axis; e.g., amplitude = 4 cm).

RESULTS AND DISCUSSION

The use of NSCs to address the widespread oligodendroglial pathology of the *shi* CNS was predicated on three observations: (*i*) our previous determination that NSC clones are capable of differentiating into morphologically, immunocytochemically, and ultrastructurally proven oligodendrocytes *in vitro* and *in vivo* after transplantation into wild-type mouse brain (2, 6, 24, 25, 27); (*ii*) our confirmation at the outset of these experiments that they are capable of producing MBP (Fig. 1); and (*iii*) our prior experience that the implantation and integration of exogenous NSC clones into germinal zones of fetuses and newborns (1, 2, 26, 27) could ensure their dissemination throughout a recipient's brain, with normal development of the virtually chimeric regions to which they contribute.

Therefore, clonal NSCs were transplanted at birth into the brains of *shi* mice (as well as unaffected controls), using the same intracerebroventricular implantation technique devised previously for the widespread engraftment of enzyme-expressing NSCs to treat global metabolic lesions (1, 2). Within 24 hr after implantation, consistent with our prior observations (1, 2, 27),

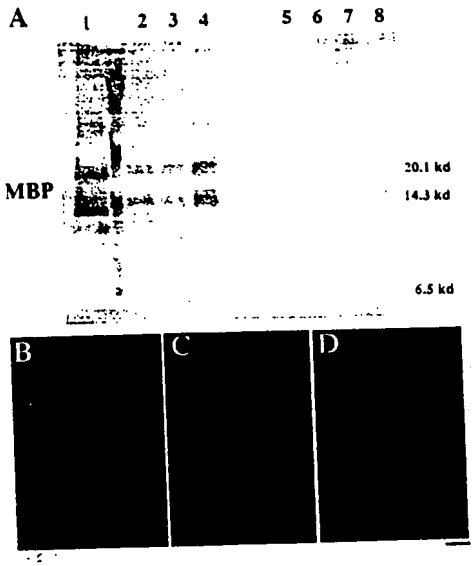

FIG. 3. MBP expression in mature transplanted and control brains. (*A*) Western analysis for MBP in whole-brain lysates. The brains of three representative transplanted *shi* mutants (lanes 2–4) express MBP at levels close to that of an age-matched, unaffected mouse (lane 1, positive control) and significantly greater than the amounts seen in untransplanted (lanes 7 and 8, negative control) or unengrafted (lanes 5 and 6, negative control), age-matched *shi* mutants. (Identical total protein amounts were loaded in each lane.) (*B–D*) Immunocytochemical analysis for MBP. (*B*) The brain of a mature unaffected mouse is immunoreactive to an antibody to MBP (revealed with a Texas Red-conjugated secondary antibody). (*C* and *D*) Age-matched engrafted brains from *shi* mice similarly show immunoreactivity. Untransplanted *shi* brains lack MBP. Therefore, MBP immunoreactivity also classically has been a marker for normal, donor-derived oligodendrocytes (*C* and *D*) in transplant paradigms (31).

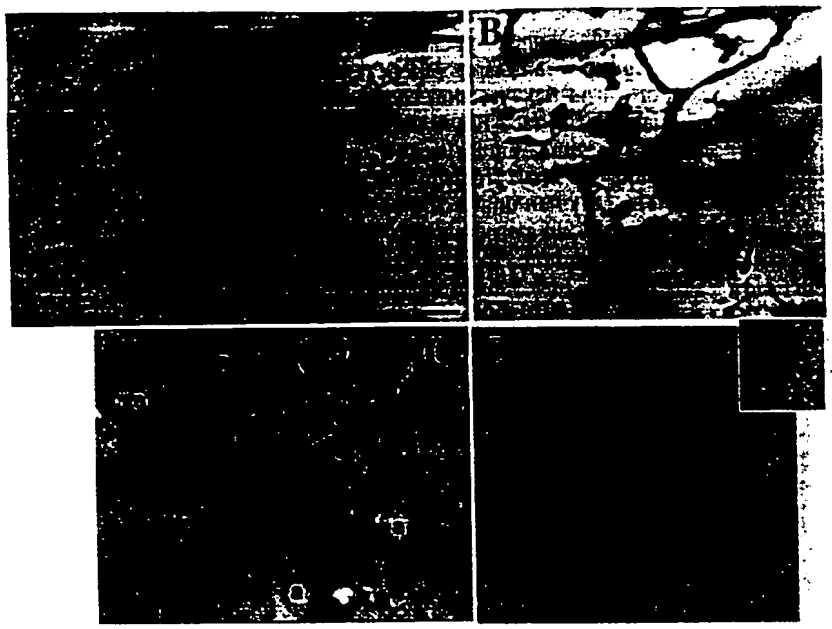

FIG. 4. Engrafted NSCs in recipient *shi* mutants differentiate into oligodendrocytes. (*A* and *B*) Donor-derived X-gal$^+$ cells in representative sections through the corpus callosum possess characteristic oligodendroglial features (small, round or polygonal cell bodies with multiple fine processes oriented in the direction of the neural fiber tracts). (*C*) Close-up of a representative donor-derived anti-β-gal immunoreactive oligodendrocyte (arrow) extending multiple processes toward and beginning to wrap large, adjacent axonal bundles ("a") viewed on end in a section through the corpus callosum. That cells such as those in *A–C* (and in Fig. 3 *C* and *D*) are oligodendroglia is confirmed by the representative electron micrograph in *D* (and in Fig. 5*A*), demonstrating the defining ultrastructural features detailed in *Materials and Methods*. A donor-derived X-gal-labeled oligodendrocyte ("LO") can be distinguished by the electron-dense X-gal precipitate that typically is localized to the nuclear membrane (arrow), endoplasmic reticulum (arrowhead), and other cytoplasmic organelles. The area indicated by the arrowhead is magnified in the *Inset* to demonstrate the unique crystalline nature of individual precipitate particles.

NSCs integrated within the subventricular zone throughout the length of the ventricular system and, by 1–2 weeks posttransplant, had migrated into and engrafted extensively within the *shi* brain parenchyma (Fig. 2). At maturity, *lacZ*$^+$ donor-derived cells were integrated seamlessly throughout the *shi* neuraxis (Fig. 2*A*), including within white tracts (Fig. 2 *B–D*). The brains of transplanted *shi* mutants, as assessed by Western analysis of whole-brain lysates (Fig. 3*A*), now expressed readily detectable levels of MBP (lanes 2–4) that contrasted markedly with the absence of MBP in unengrafted, age-matched *shi* brains (lanes 5–8) and compared favorably with that present in unaffected brains (lane 1). Immunocytochemistry analysis using an antibody to MBP (Fig. 3 *B–D*) (31) confirmed expression of MBP at the cellular level in engrafted *shi* brains (Fig. 3 *C* and *D*) with an immunoreactivity comparable to that in nonmutant brains (Fig. 3*B*). Therefore, transplantation of NSCs into the MBP-deficient *shi* brain resulted in widespread engraftment with repletion of significant amounts of whole-brain MBP.

This observation lent support to the expectation that, had donor NSCs indeed differentiated into mature, normal oligodendrocytes, then they would effectively enwrap host axons and dendrites with better-compacted myelin. The phenotype of transplanted NSCs, therefore, was confirmed by LM and EM.

Under bright field, such donor-derived, *lacZ*$^+$ cells, particularly within white tracts, indeed possessed a morphology classic for

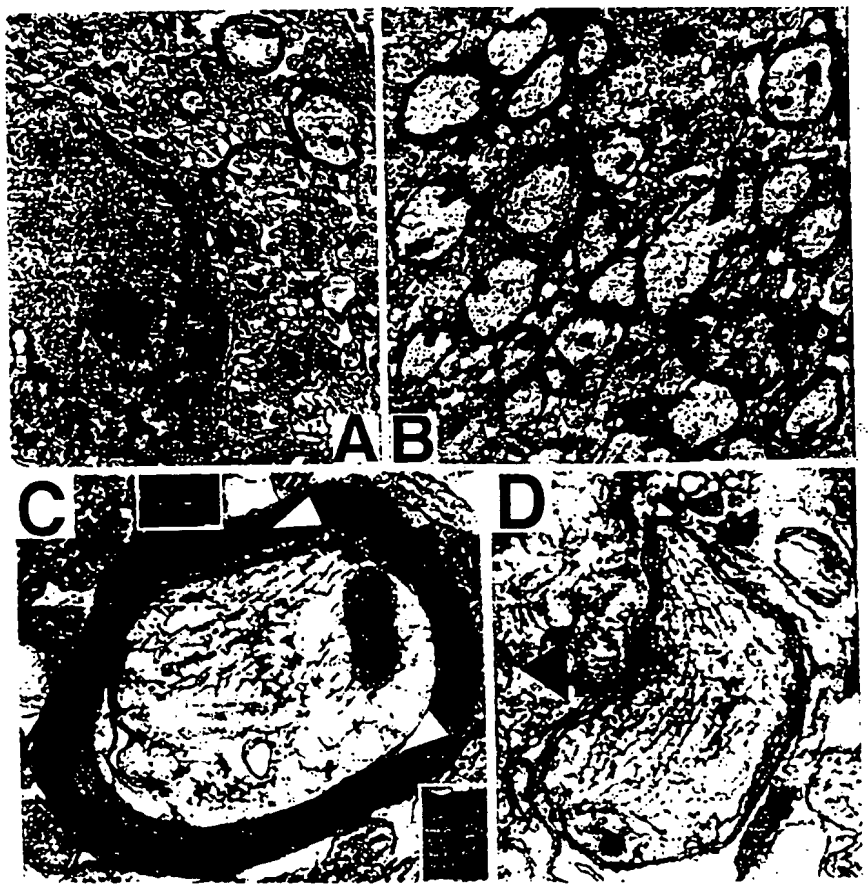

FIG. 5. NSC-derived "replacement" oligodendrocytes appear functional as demonstrated by ultrastructural evidence of myelination of *shi* axons. In regions of MBP-expressing NSC engraftment, *shi* neuronal processes become enwrapped by thick, better-compacted myelin. (*A*) At 2 weeks posttransplant, a representative donor-derived, labeled oligodendrocyte ("LO") [recognized by extensive X-gal precipitate ("p") in the nuclear membrane, cytoplasmic organelles, and processes] is extending processes (a representative one is delineated by arrowheads) to host neurites and is beginning to ensheathe them with myelin ("m"). (*B*) If engrafted *shi* regions, such as that in *A*, are followed over time (e.g., to 4 weeks of age as pictured here), the myelin begins to appear healthier, thicker, and better compacted (examples indicated by arrows) than that in age-matched, untransplanted control mutants. (*C*) By 6 weeks posttransplant, these mature into even thicker wraps; ≈40% of host axons are ensheathed by myelin (a higher-power view of a representative axon is illustrated in *C*) that is dramatically thicker and better compacted than that of *shi* myelin [an example of which is shown in *D* (black arrowhead) from an unengrafted region of an otherwise successfully engrafted *shi* brain]. In *C*, open arrowheads indicate representative regions of myelin that are magnified in the adjacent respective *Insets*; major dense lines are evident.

Table 1. Morphometric and behavioral analysis

Parameter	Normal	Shiverer	
		Unengrafted	Engrafted
Neuronal processes with myelin and MDLs,* %	96.7	0	37.8
Periodicity of myelin,† nm	4.5 ± 0.2	24.4 ± 5.8	10.5 ± 0.7
Width of myelin wrap, nm	138 ± 5	59.5 ± 1.5	135 ± 20
Degree of tremor (as tail displacement,‡ cm	0	4	1.2 ± 1.6
Behavioral score§ (scale = 1–4)	3.91 ± 0.14	2.09 ± 0.08	3.72 ± 0.20

*MDLs, major dense lines, an indication of compacted myelin; the data represent the mean percentage of axons and dendrites with MDLs in representative specimens examined.

†See *Materials and Methods* for definition. The shorter the distance, the better compacted and, hence, more normal the myelin.

‡See "Behavioral Assessment" in *Materials and Methods* as well as Fig. 6 *C* and *D* for a description. Zero centimeter of tail displacement suggests minimal to no tremor; 4 cm of displacement reflects extensive tremor. Of the 10 transplanted *shi* mice examined, 6 actually showed zero displacement.

§See "Behavioral Assessment" in *Materials and Methods* as well as Fig. 6 *A* and *B* for a description of the scoring system. Unengrafted *shi* mutants scored significantly worse than normal mice ($P < 0.0001$); the scores for successfully engrafted *shi* mice examined in this fashion, however, ($n = 6$) were statistically indistinguishable from those of normal mice ($P = 0.20$) and significantly better than unengrafted *shi* mice ($P < 0.0001$). Unsuccessfully transplanted *shi* mice ($n = 4$; mean score = 1.97 ± 0.29) were indistinguishable from untransplanted *shi* [i.e., significantly different from scores in the "normal" column ($P < 0.0003$), not statistically different from scores in the "unengrafted *shi*" column].

oligodendrocytes (Fig. 4 *A–C*), typically extending processes toward large axonal bundles (Fig. 4*C*). The crystalline X-gal precipitate is electron-dense, ensuring unambiguous designation and characterization of donor-derived cells even at the EM level (8, 24–30). EM analysis of x-gal+ donor-derived cells confirmed that they met the defining ultrastructural criteria of oligodendrocytes (e.g., Figs. 4*D* and 5*A*) (detailed in *Materials and Methods*).

Therefore, donor NSCs could differentiate into ultrastructurally confirmed oligodendrocytes in the engrafted *shi* brain. Of interest was the additional observation that, although these multipotent donor cells were able to differentiate into multiple neural cell types in the engrafted *shi* brains, a significantly greater percentage of engrafted NSCs differentiated toward an oligodendroglial phenotype in the *shi* brain (28%) than in normal controls (16%; $\chi^2 = 0.015$), suggesting that NSCs actually may be compensating somewhat specifically for oligodendrocyte dysfunction in *shi*. Of note, a similar shift in the fate of this same clone of multipotent NSCs toward a neuronal phenotype was detected in developing (26) and adult (25) mouse brain when that neural cell type was deficient or defective and required compensation. Taken together these observations suggest that NSCs might possess a mechanism whereby their differentiation is directed to replenish deficient or inadequate cell types. Such behavior may reflect a fundamental developmental strategy with therapeutic utility.

The successful repletion of MBP in the *shi* brain suggested that donor-derived oligodendrocytes should be functional and, hence, form healthier myelin throughout the brain. Indeed, as early as 2 weeks posttransplant, a subpopulation of donor-derived oligodendrocytes extended processes that enwrapped host axons and dendrites and began laying down myelin around neuronal processes (Fig. 5*A*). Over a period of 3–4 weeks, the myelin produced by these oligodendrocytes appeared healthier, thicker, and better compacted (Fig. 5*B*). By 6 weeks posttransplant, a mean of ≈40% of host neuronal processes (Table 1) (up to 52% in some representative specimens examined) were ensheathed by donor-derived myelin (Fig. 5*C*) that contrasted dramatically with that observed in untransplanted control mutants or even with that in unengrafted areas of successfully transplanted *shi* animals (Fig. 5*D*) (an internal control for the efficacy of engraftment) and that compared quite favorably with wild-type myelin. Morphometric analysis of myelinated neuronal processes in engrafted mutants confirmed that the periodicity of myelin was significantly closer to and the mean thickness of myelin virtually equaled ($P > 0.1$) that of normal controls (Table 1).

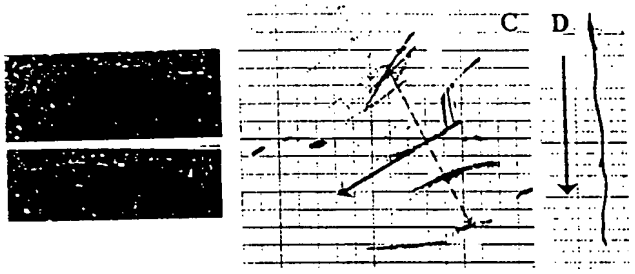

FIG. 6. Functional and behavioral assessment of transplanted *shi* mutants and controls. The *shi* mutation is characterized by the onset of tremor and a "shivering gait" by the second to third postnatal week. The degree of motor dysfunction in animals was gauged in two ways: (*i*) by blindly scoring periods of standardized videotaped cage behavior of experimental and control animals and (*ii*) by measuring the amplitude of tail displacement from the body's rostral–caudal axis (an objective, quantifiable index of tremor) (see *Materials and Methods*). Video freeze-frames of representative unengrafted and successfully engrafted *shi* mice are seen in *A* and *B*, respectively. The whole-body tremor and ataxic movement observed in the unengrafted symptomatic animal (*A*) causes the frame to blur, a contrast to the well focused frame of the asymptomatic transplanted *shi* mouse (*B*). The neurologic scoring of such mice is detailed in *Materials and Methods* and Table 1; 60% of transplanted mutants evinced nearly normal-appearing behavior as in *B* and attained scores that were not significantly different from normal controls. *C* and *D* depict the manner in which whole-body tremor was mirrored by the amplitude of tail displacement (hatched, gray arrow in *C*), measured perpendicularly from a line drawn in the direction of the animal's movement (solid, gray arrow, which represents the body's long axis) (see *Materials and Methods*). Measurements were made by permitting a mouse, whose tail had been dipped in India ink, to move freely in a straight line on a sheet of graph paper as shown. Large degrees of tremor cause the tail to make widely divergent ink marks away from the midline, representing the body's axis (*C*). Absence of tremor allows the tail to make long, straight, uninterrupted ink lines on the paper congruent with the body's axis (*D*). The distance between points of maximal tail displacement from the axis was measured and averaged for transplanted and untransplanted *shi* mutants and for unaffected controls (hatched, gray arrow). *C* shows data from a poorly engrafted mutant that did not improve with respect to tremor whereas *D* reveals lack of tail displacement in a successfully engrafted, now asymptomatic *shi* mutant. Overall, 64% of transplanted *shi* mice examined displayed at least a 50% decrement in the degree of tremor or "shiver." Several showed zero displacement (see Table 1).

The success of NSC transplantation in *shi* ultimately is determined by its ability to achieve functional improvement. To this end, transplanted mutants as well as unaffected (positive control) and untransplanted *shi* (negative control) mice were analyzed (as detailed in *Materials and Methods*) for functional improvement by (*i*) scored neurologic assessment during free-cage behavior and (*ii*) quantifying the degree of "shiver" as reflected in the amplitude of tail tremor. Behaviorally relevant tremors were decreased significantly in a significant number of representative recipient mutants (Fig. 6 and Table 1): 60% of tested transplanted mutants evinced behavior that approximated normal (Fig. 6*B*), i.e., attained neurologic scores that both individually and as a group mean were statistically indistinguishable from normal controls ($P > 0.1$) on the behavioral scale detailed in *Materials and Methods* (Table 1); 64% of transplanted animals showed at least a 50% decrement in measured tremor, and some engrafted animals evinced virtually no "shiver" (and, hence, essentially no

tail displacement) (Fig. 6*D*). This suggests that the "replacement cells" (in this case, oligodendrocytes) were integrated into host CNS in a functionally relevant manner.

The variability in behavioral improvement after engraftment does not have a simple explanation: there did not appear to be a simple correlation between functional improvement and the degree of NSC engraftment or MBP expression. Etiology of the "shivering" phenotype, however, is complex and not well understood. Symptomatic improvement may not be simply a measure of the overall amount of successful myelination. Instead, it may be more reflective of successful remyelination in specific CNS regions. Indeed, one hypothesis holds that there is a shivering "center" affected in *shi* mice. If this hypothesis holds, variations in experimental animals may represent the degree to which such a center was myelinated successfully. It is also important to note that we did not focus on addressing spinal cord defects in *shi*; these lesions likely mediate expression of symptoms as well. Despite the fact that we obtained significant NSC engraftment, MBP expression, oligodendrocyte differentiation, and myelination, it is likely that these could be optimized further: given that NSCs are so readily manipulated (5–8), future studies could use NSCs genetically engineered or pretreated (3, 6) *ex vivo* to enhance these capacities. It is also unclear which role donor-derived nonoligodendroglial cells might have played in effecting repair of the dysmyelinated *shi* brain. Neurons and astrocytes have been implicated in oligodendrocyte migration and differentiation and may influence myelination. The extent to which such other neural cell types, derived from the same clone of NSCs, may have had "helper" roles in improvement might constitute an additional argument for the use of multipotent cells rather than those with a more restricted fate. Indeed, many diseases—even those classically characterized as purely disorders of white or gray matter—actually affect a mixed population of cell types and would benefit from the concurrent replacement of both neuronal and glial elements.

Conclusions

Transplanted NSCs can differentiate into MBP-expressing oligodendrocytes throughout the *shi* brain, in turn promoting improved widespread remyelination with extensive amelioration of neuropathology and symptoms. These results suggest that NSCs may be useful for a variety of diseases characterized by profuse white matter degeneration that might benefit from the replacement of oligodendroglia. Disordered myelination plays an important role in many other genetic and acquired neurodegenerative processes. In a broader sense, with oligodendroglia serving as a model for neural cells in general and *shi* serving as a prototype for a broad range of maladies characterized by extensive neural cell dysfunction, these results suggest that therapeutic cell replacement in a widely disseminated, even "global," manner is feasible when cells with stem-like qualities are used as graft material, a recognition that broadens the paradigmatic scope of neural transplantation. Furthermore, an NSC-based approach, whether with exogenous NSCs or with appropriately mobilized endogenous NSCs, may address therapeutic challenges that previously have been inaccessible. Many promising gene therapy vehicles and biochemical treatment modalities depend on relaying "new" genetic information through "old" neural substrates that may, in fact, have been lost, become dysfunctional, or failed to develop. Reconstitution of aspects of this anatomy may enable more significant recovery. Furthermore, although the ability of NSCs to engraft diffusely has been exploited for widespread distribution of enzymes (1, 2) and, now, cells, it seems apparent that a similar strategy can be used for dissemination of a variety of diffusible (e.g., neurotrophins, viral vectors) (33) and nondiffusible (e.g., extracellular matrix) factors for a wide range of therapeutic and research demands. Combined therapies may forestall damage while restoring lost neural elements. NSCs with similar properties recently have been generated from human tissue, laying the groundwork for potential treatments of heretofore refractory human diseases (27, 34). The persistence of a periventricular zone in the human for prolonged periods postnatally suggests that implantation strategies similar to those described may be feasible.

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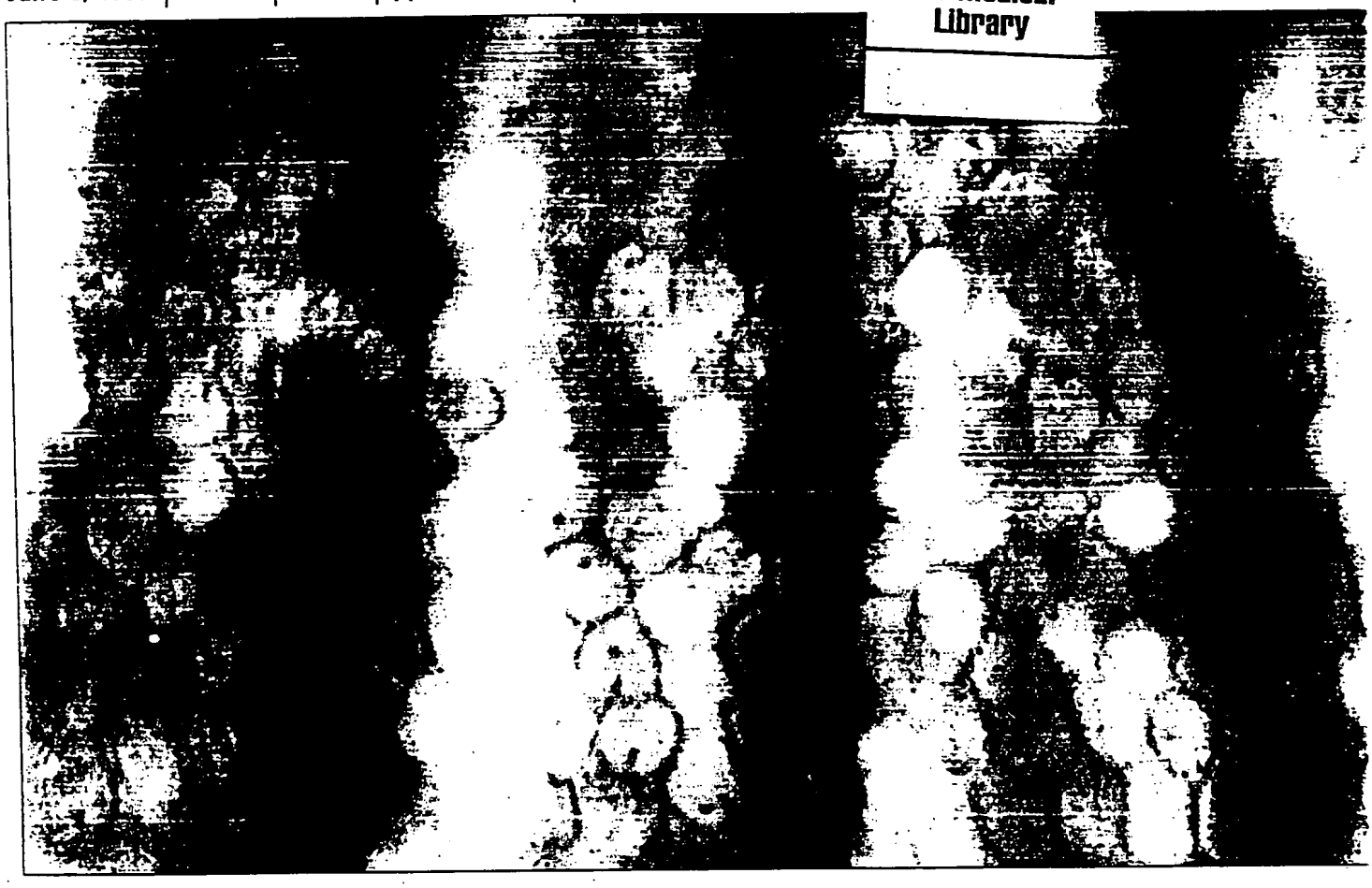

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Site-Specific Migration and Neuronal Differentiation of Human Neural Progenitor Cells after Transplantation in the Adult Rat Brain

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Neural progenitor cells obtained from the embryonic human forebrain were expanded up to 10^7-fold in culture in the presence of epidermal growth factor, basic fibroblast growth factor, and leukemia inhibitory growth factor. When transplanted into neurogenic regions in the adult rat brain, the subventricular zone, and hippocampus, the *in vitro* propagated cells migrated specifically along the routes normally taken by the endogenous neuronal precursors: along the rostral migratory stream to the olfactory bulb and within the subgranular zone in the dentate gyrus, and exhibited site-specific neuronal differentiation in the granular and periglomerular layers of the bulb and in the dentate granular cell layer. The cells exhibited substantial migration also within the non-neurogenic region, the striatum, in a seemingly nondirected manner up to ~1–1.5 mm from the graft core, and showed differentiation into both neuronal and glial phenotypes. Only cells with glial-like features migrated over longer distances within the mature striatum, whereas the cells expressing neuronal phenotypes remained close to the implantation site. The ability of the human neural progenitors to respond *in vivo* to guidance cues and signals that can direct their differentiation along multiple phenotypic pathways suggests that they can provide a powerful and virtually unlimited source of cells for experimental and clinical transplantation.

Key words: progenitor cells; human; transplantation; neuron; subventricular zone; dentate gyrus; striatum

The limited capacity for structural repair in the mammalian brain is in part explained by the inability of the mature CNS to generate new cellular elements in response to damage. Cell transplantation offers a possibility to circumvent this limitation. Both rodent and primate experiments show that neuroblasts and young postmitotic neurons obtained from defined parts of the neuraxis during development can survive, mature, and grow extensive functional axonal connections after transplantation to brain-damaged recipients, and both structurally and functionally replace lost neurons in the mature brain (for review, see Dunnett and Björklund, 1994). Because of the limited migratory capacity of the differentiated cells, however, these types of implants are unable to integrate into the cellular architecture of the host.

Previous studies have shown that less differentiated precursor cells, taken at premigratory stages of neuronal development, can make use of available substrates or pathways for migration, mix with endogenous pools of precursors, and participate in ongoing neurogenesis, both during development (McConnell, 1988; Gao and Hatten, 1994; Zigova et al., 1996) and in areas of the mature brain, the anterior subventricular zone (SVZa), and the hippocampal dentate gyrus, where neurogenesis continues into adulthood (Lois and Alvarez-Buylla, 1993; Vicario-Abejon et al., 1995). Similarly, mixed precursor cell populations, injected *in utero* into the developing forebrain, integrate across the ventricular wall and undergo site-specific migration and neuronal differentiation in widespread brain regions (Brüstle et al., 1995; Campbell et al., 1995; Fishell, 1995), suggesting that undifferentiated progenitors may be an interesting source of cells for intracerebral transplantation.

Recently, neural progenitors with the capacity to give rise to all major cell types of the mature CNS have been isolated from the developing or adult CNS (Weiss et al., 1996b; Alvarez-Buylla, 1997; Luskin et al., 1997; Ray et al., 1997). They become more restricted in number during development and remain as a small, relatively quiescent population of dividing cells in the subventricular regions of the adult CNS. These neural progenitors can be grown *in vitro* in the presence of either epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF, FGF-2), as a population of continuously dividing progenitors capable of differentiating into both neurons and glia (Murphy et al., 1990; Reynolds and Weiss, 1992a,b, 1996; Richards et al., 1992; Ray et al., 1993; Vescovi et al., 1993; Sensenbrenner et al., 1994; Palmer et al., 1995). Cells isolated from the rat hippocampus in the presence of bFGF have been shown to express region-specific migration and neuronal differentiation after transplantation to the adult rat brain (Gage et al., 1995; Suhonen et al., 1996). Embryonic mouse or rat forebrain progenitors expanded in the presence of EGF, by contrast, develop into predominantly glial phenotypes *in vivo*, as observed after transplantation to the adult rat spinal cord (Hammang et al., 1997) or the developing rat forebrain (Winkler et al., 1998).

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Here, we have examined the question of whether progenitors isolated from the developing human CNS can exhibit *in vivo* neurogenic properties after implantation into the brain of adult recipients. Cells obtained from the forebrain of 6.5- to 9-week-old human fetuses were maintained as continuously dividing cultures in the presence of EGF, bFGF, and leukemia inhibitory growth factor (LIF). Cells expanded 10^3-10^7-fold in culture (over 9–21 passages) survived well after transplantation to both neurogenic and non-neurogenic sites; cells contained within these grafts showed migration, integration, and site-specific differentiation into both neurons and glia.

MATERIALS AND METHODS

Generation and in vitro culture of human progenitor cells. Generation of the human progenitor cell lines has been described previously (Carpenter et al., 1999). Embryonic brain tissue was obtained from one 6.5 week and one 9 week embryo (post-conception) under compliance with National Institutes of Health guidelines, Swedish government guidelines, and the local ethics committee, and appropriate consent forms were used. Tissue from the forebrain was dissected in sterile saline and transferred to N2 medium, a defined DMEM/F12-based medium (Life Technologies, Grand Island, NY) containing 0.6% glucose, 25 μg/ml human insulin, 100 μg/ml human transferrin, 20 nM progesterone, 60 μM putrescine, 30 nM selenium chloride, 2 mM glutamine, 3 mM sodium bicarbonate, 5 mM HEPES, and 2 μg/ml heparin (Sigma, St. Louis, MO). The tissue was dissociated using a standard glass homogenizer, and the dissociated cells were grown on uncoated plastic T75 flasks in N2 medium containing human EGF (hEGF, 20 ng/ml; Life Technologies), human basic FGF (hbFGF, 20 ng/ml; Life Technologies), and human LIF (hLIF) (10 ng/ml, R+D Systems, UK), at a density of ~100,000 cells/ml.

The cells grew as free-floating clusters ("neurospheres"), and were prevented from attachment by gently knocking the flasks each day. Any cells that adhered to the plastic and began to extend processes were not removed by this procedure and therefore were not carried through to the next passage. The spheres were passaged by mechanical dissociation every 7–10 d and reseeded as single cells at a density of ~100,000 cells/ml. The cells used for transplantation (the 6.5FBr and 9FBr cultures) had been expanded over 9–21 passages, which corresponds to a total increase in cell numbers of ~10^9 at 9 passages to at least 10^7 at 21 passages (Carpenter et al., 1999).

Labeling methods and preparation of cells for transplantation. To enable the detection of the cells *in vivo*, cultures were labeled with 1 μM bromodeoxyuridine (BrdU), which was added to the culture medium 48 hr before the preparation of the cells for transplantation. This resulted in ~80% labeling efficiency, with no apparent changes in growth rate of the spheres.

Cells were taken for transplantation 4–5 d after the last passage as small spheres of 5–30 cells. The spheres were collected by centrifugation at 1000 rpm for 3 min and resuspended in 1 ml DMEM/F12 medium. To check the cell viability, an aliquot of the sphere suspension was removed and mixed with trypan blue. After this was ascertained, a second cell count was performed by triturating the trypan blue aliquot to give single cells. The sphere suspension was centrifuged a second time and resuspended in a smaller volume to give the equivalent of ~100,000 cells/μl.

Transplantation. Adult female Sprague Dawley rats (B&K Universal, Stockholm, Sweden), weighing ~250 gm at the beginning of the study, were used. They were caged in groups of two and maintained on a 12 hr light/dark cycle with constant temperature and humidity, with *ad libitum* food and water. The animals were immunosuppressed throughout the experiment by daily injections of 10 mg/kg cyclosporin, beginning 1 d before transplantation.

Stereotaxic surgery was performed under deep equithesin anesthesia (3 ml/kg body weight, i.p.). Rats received 1 μl cell suspension bilaterally in either the SVZa, rostral migratory stream (RMS), or hippocampus, or 2 μl in the striatum, according to the following coordinates: SVZa, anterior (A) = +1.6, lateral (L) = ±1.5, ventral (V) = −4.2; RMS, A = −3.7, L = ±1.5, V = −5.0; hippocampus, A = −3.6, L = ±2.0, V = −3.0, −2.6; striatum, A = 0.6, L = ±2.8, V = −4.8, −4.2. The tooth bar was set at −2.3, and all ventral coordinates were taken from dura. Cells were implanted via a glass capillary (inner diameter ~70 μm) attached to a 2 μl Hamilton syringe. For the SVZa transplants, 100,000 cells from the 6.5FBr cell line were transplanted, and the brains were analyzed after 6 weeks (n = 10). For the RMS transplants, 100,000 cells were transplanted, and the brains were analyzed at either 2 weeks (6.5FBr, n = 10; 9FBr, n = 4) or 6 weeks (6.5FBr, n = 10). Both cell lines were transplanted to either the striatum (200,000 cells) or hippocampus (100,000 cells), and the brains were analyzed at either 2 or 6 weeks (n = 10 per group).

Tissue processing. At either 2 or 6 weeks after transplantation, rats were terminally anesthetized with 5% chloral hydrate and transcardially perfused with 0.1 M PBS followed by 5 min rapid fixation with ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. Brains were removed and placed in PFA overnight, before being transferred to 25% sucrose in PBS. Coronal or sagittal sections were cut on a freezing microtome at a thickness of 30 μm. In each case, eight series were collected for further processing.

Immunocytochemistry. For BrdU labeling, all sections were pretreated with 1 M HCl for 30 min at 65°C. Sections were incubated in primary antibodies for 36 hr at 4°C. All primary antibodies were diluted in 0.02 M potassium PBS (KPBS) containing 3% normal serum of the species in which the secondary antibody was raised and 0.25% Triton X-100, except for 32 kDa dopamine- and cAMP-regulated phosphoprotein (DARPP-32) and L-amino decarboxylase (DAD_{67}) in which Triton X-100 was omitted. Antibodies used in this study were BrdU rat monoclonal (1:100, Chemicon, Temecula, CA), mouse monoclonal (1:25, Becton Dickinson, Franklin Lakes, NJ), β-tubulin-III (1:400, Sigma), calbindin (1:1000, Sigma), DARPP-32 (1:20,000; Dr. P. Greengard, Rockefeller), GAD_{67} (1:1000, Chemicon), glial fibrillary acidic protein (GFAP, 1:500, Dakopatts), RNA binding protein (Hu, 1:1000; Dr. S. Goldman, Cornell), neuronal nuclei (NeuN, 1:100, Chemicon), tyrosine hydroxylase (TH, 1:500, PelFreeze, Rogers, AR), Vimentin (VIM, 1:25, Dakopatts), and human-specific tau (hTau, 1:100, Calbiochem, La Jolla, CA). For all immunohistochemical procedures, adjacent sections served as negative controls and were processed using identical procedures, except for incubation without the primary antibody in each case.

For fluorescent double-labeling immunocytochemistry, after rinses in KPBS containing 2% of the normal sera, sections were incubated in the secondary antibodies (1:200). For rat anti-BrdU this was donkey anti-rat conjugated to FITC or Cy2 (Jackson); for mouse anti-BrdU, donkey anti-mouse conjugated to FITC or Cy2 (Jackson); for all other primary antibodies raised in mouse, rat-absorbed biotinylated horse anti-mouse (Vector); and for all primary antibodies raised in rabbit, biotinylated swine anti-rabbit (Dakopatts). All secondaries were diluted in KPBS containing 2% normal serum, and sections were reacted for 2 hr at room temperature in the dark. After three rinses in KPBS, sections were reacted with streptavidin conjugated to Cy3 (Jackson) for a further 2 hr at room temperature in the dark.

For immunohistochemistry with hTau, sections were pretreated with 3% H_2O_2 in 10% methanol to quench endogenous peroxidase activity. Incubation in the primary antibody was performed in KPBS containing 5% normal horse serum and 0.25% Triton X-100 for 36 hr at 4°C. After three rinses in KPBS, sections were incubated in the secondary antibody: rat-absorbed biotinylated horse anti-mouse (Vector) in KPBS containing 2% normal horse serum for 2 hr at room temperature. Further washing in KPBS was followed by incubation with avidin–biotin–peroxidase complex (Vectastain, Vector), for 1.5 hr at room temperature. 3,3-Diaminobenzidine (Sigma) in 0.03% H_2O_2 in KPBS was used as the chromogen.

The sections were mounted on chrome–alum-coated slides, and the fluorescent sections were coverslipped using polyvinyl alcohol–1,4-diazabicyclo[2,2,2]octane mounting medium. The hTau slides were dehydrated in ascending alcohols and coverslipped using DPX mountant.

Confocal microscopy. Colocalization of BrdU with neuronal and glial markers was conducted by confocal microscopy to enable exact definition of each of the antibodies, using a Bio-Rad MRC1024UV confocal scanning light microscope. Double-labeled cells were always verified, both by collecting serial sections of 1–2 μm throughout the specimen, and by eye, using an Olympus binocular microscope. In all figures, all double-labeled cells that are denoted were identified in this way.

RESULTS

In vitro characteristics of the transplanted cells

Two different human progenitor cell cultures obtained post mortem from the forebrain of one 6.5 week (6.5FBr) and one 9 week (9FBr) embryo were analyzed. The cells were cultured in the presence of EGF, bFGF and LIF and passaged every 7–10 d. In

these cultures bFGF was necessary to maintain continuous cell proliferation over extended time periods, and this effect was further enhanced by the addition of LIF. Parallel *in vitro* experiments (Carpenter et al., 1999) indicate that LIF promotes the sustained proliferation of the human progenitors in the neurosphere cultures. Moreover, in agreement with previous findings (Satoh and Yoshida, 1997), the proportion of cells that differentiated into neurons appeared to be increased in the presence of LIF.

The *in vitro* characteristics of the 6.5FBr and 9FBr progenitor cell cultures have been presented in detail elsewhere (Carpenter et al., 1999). Briefly, both cultures showed a growth rate that was similar to each other and to other human progenitor cell cultures derived from different gestational ages. Cells within undifferentiated spheres were immunopositive for the immature cell marker nestin and were shown to incorporate BrdU, indicative of cell division. To assess the differentiation capacity of these cells, dissociated single cells were plated onto poly-ornithine-coated glass coverslips and cultured for 12–14 d in N2 medium containing 1% FBS. On differentiation, both cell cultures demonstrated the capacity to form neurons, astrocytes, and oligodendrocytes. Immunohistochemistry using an antibody to GFAP revealed a range of 15–55% astrocytes present in both the 6.5FBr and 9FBr cultures between passage 5 (P5) and P35. An antibody to β-tubulin isotype III was used to detect neurons. At P5 the 6.5FBr cultures generated more β-tubulin-III-positive cells than the 9FBr cells (37 vs 20%, respectively). At P20–P30 (150–300 d *in vitro*), the percentage of neurons had decreased to ~15% in both cultures.

Survival and differentiation after transplantation to the adult rat brain

Cells from the 6.5FBr and 9FBr cultures were transplanted, under immunosuppression, into two neurogenic sites: the dentate gyrus of the hippocampus and the SVZa and its associated RMS, as well as to a non-neurogenic site, the striatum. Transplantation was performed using cells that had been passaged 9–21 times. The cells were labeled with BrdU during the last 48 hr before transplantation. This resulted in ~80% labeling efficiency and enabled analysis of the grafts by fluorescent immunohistochemistry using a double-labeling technique for BrdU in combination with specific neuronal and glial markers. In addition, hTau was used to identify the grafted cells.

In all animals, BrdU-positive transplanted cells were identified in all graft sites, at both 2 and 6 weeks after transplantation. Similarly, staining with the human-specific tau antibody revealed cellular and axonal profiles at all transplant sites, indicating graft survival in all cases. Extensive migration of BrdU-labeled cells, as described below, were seen in all animals where the graft deposits had been correctly placed in the RMS, SVZa, or hippocampus, respectively. No evidence of tumor formation was observed.

The transplants from both cell cultures (6.5FBr and 9FBr), regardless of the number of passages, were indistinguishable in terms of graft survival, migrational patterns, and phenotypic differentiation of the transplanted cells. Control transplants of cells that had been killed by freeze-thawing before transplantation showed no transfer of the BrdU marker to the host cells, which is in agreement with previous reports (Gage et al., 1995; Suhonen et al., 1996).

The subventricular zone and rostral migratory stream

Single deposits of 100,000 cells were deposited in or close to the SVZa, just ventral to the corpus callosum, or just above the RMS midway between the SVZa and the olfactory bulb. In the SVZa a core of BrdU-positive cells was located close to the ventricular ependyma, extending in some cases into the white matter of the overlying corpus callosum (Fig. 1*A*) (6 weeks survival). Cells were seen to leave the transplantation site in a stream of rostral migration (Fig. 1*B*) after the RMS, i.e., along the path of endogenous progenitors toward the olfactory bulb. Once they reached the bulb, BrdU-positive cells left the migratory stream, becoming dispersed throughout the subependymal, granular (Fig. 1*C*,*D*), and glomerular cell layers. The cells within the olfactory bulb were more weakly BrdU-labeled than the cells in the SVZa (which were uniformly highly labeled), suggesting that the labeled cells had undergone further cell division on their route to the bulb, similar to the endogenous progenitors from the SVZa (Menezes et al., 1995).

In the RMS transplants the deposits of BrdU-labeled cells were localized just above, and occasionally within, the RMS itself (see Fig. 4*A*). At 2 weeks after transplantation the cells remained clustered at the graft site, and there was very little migration from the graft core. Thus only few cells were observed rostral and caudal to the graft placement at this time point. Six weeks after grafting, cells were seen to have migrated rostrally toward the olfactory bulb (see Fig. 4*B*) and into the granular and periglomerular layers (see Fig. 4*C*–*F*).

The immature cell marker VIM was used to delineate the SVZa and RMS along which BrdU-positive cells were seen in their migratory stream (Fig. 2*A*). BrdU-positive cells were not VIM positive. The vast majority of the BrdU-labeled nuclei did not diverge from the RMS; however, in the region adjacent to the transplant core, occasional cells could be seen migrating dorsally toward the overlying cortex (data not shown). Some of the cells migrating within the RMS were double-labeled with the early neuronal markers Hu (Fig. 2*B*) and β-tubulin-III (see Fig. 4*B*). Both of these markers, which identify both early differentiated neuronal precursors and mature neurons, are known to be expressed by the endogenous progenitors from the SVZa as they migrate along the RMS (Baraban et al., 1995; Menezes et al., 1995). The presence of these markers thus indicates their early commitment to a neuronal phenotype. None of the BrdU-positive cells within the SVZa or RMS stained positively for the NeuN marker. Within the olfactory bulb, the majority of BrdU-labeled cells, both in the deeper layers and in the periglomerular layer, were Hu positive (Figs. 2*C*, 4*C*), and approximately half of the BrdU-positive cells were also double-labeled with the more mature neuronal marker NeuN (Fig. 2*D*,*E*), indicating a progressive maturation of the cells toward a neuronal phenotype as they entered the bulb. Many of the BrdU-labeled cells, within both the granule cell layer and periglomerular layer, also expressed the GABA-sythesizing enzyme GAD_{67} (see Fig. 4*D*,*E*). TH, which is a characteristic feature of the dopaminergic periglomerular neurons, was clearly expressed in some of the BrdU-labeled cells within the periglomerular layer (Fig. 4*F*, *arrowheads* and *inset*). None of the BrdU-labeled cells stained positively for the glial marker GFAP, neither within the astrocyte-rich RMS (see Fig. 2*A*) nor within the olfactory bulb (see Fig. 9*B*). In addition, no cells were double-labeled with BrdU and the receptor phosphoprotein DARPP-32, which is present in the medium spiny neurons of the striatum but not normally expressed in neurons of the olfactory bulb. Table 1 gives a semiquantitative summary of neuronal and glial differentiation of the transplanted cells within the RMS and the olfactory bulb.

Using a human-specific antibody to the cytoskeletal protein

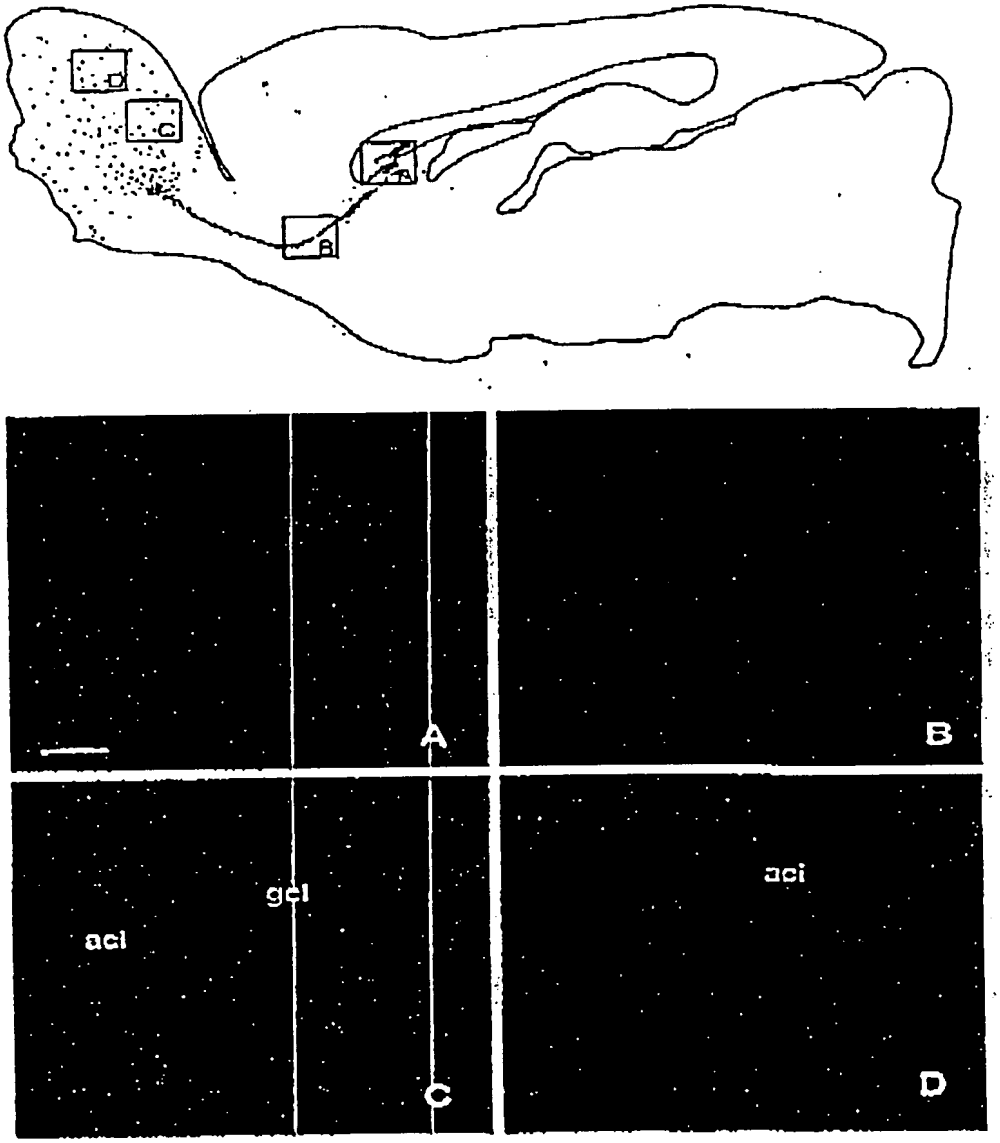

Figure 1. Low-power illustration of a transplant in the SVZa in a sagittal section, analyzed at 6 weeks after transplantation, shows an overview of the injection site of BrdU-labeled cells (*green*) and their distribution throughout the RMS. In the olfactory bulb, the cells were found dispersed through all layers. *A–D*, Grafted cells at different sites (indicated in the *top panel*), with BrdU-labeled cells shown in *green* and the NeuN shown in *red*. Double-labeled cells present in the bulb display a *yellow color* (*C*, *D*). *A*, Transplant core; *B*, cells migrating along the RMS; *C*, *D*, cells in the granule cell layer of the olfactory bulb. Scale bar, 250 μm. *aci*, Intrabulbar portion of the anterior commissure; *gcl*, granule cell layer.

tau, positive staining was observed at the injection site in both cellular and axonal profiles (Fig. 3*A*). Typically, cells that remained at the graft core or migrated only a short distance from the implantation site had developed axons that projected laterally into either the corpus callosum or striatum adjacent to the transplant (Fig. 3*A*,*B*). Tau-positive cells were distributed along the RMS, several millimeters from the graft site (Fig. 3*C*,*D*). These cells often showed a short leading process, oriented in the direction of the RMS (Fig. 4). Small tau-positive profiles were observed in the deeper layers of the olfactory bulb, and occasionally mature cells with extensive processes were found in this region (Fig. 3*E*,*F*). High background from the immunohistochemical procedure precluded the identification of tau-positive profiles in the periglomerular layer.

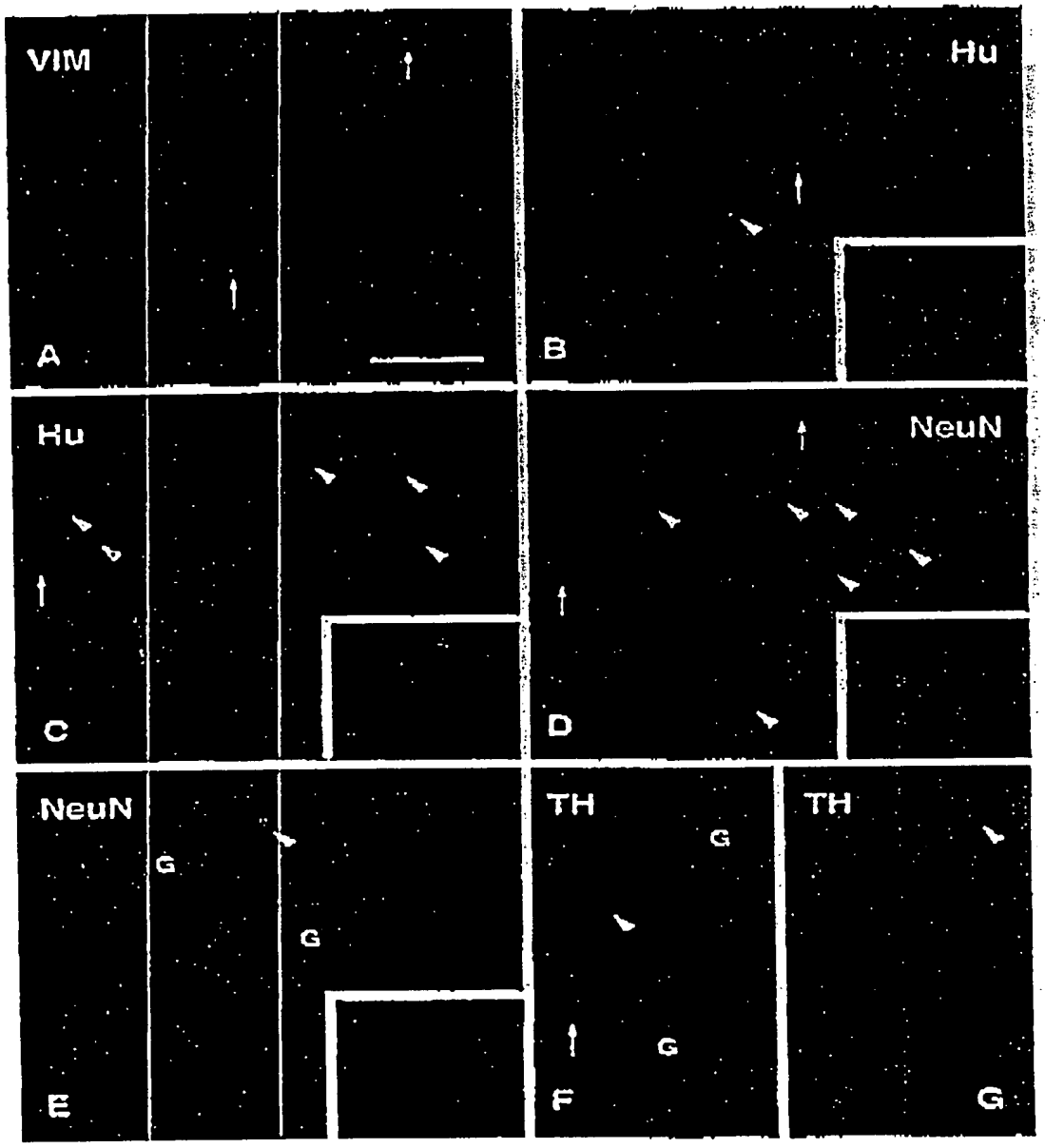

Figure 2. Confocal images of BrdU-labeled (*green*) and double-labeled cells (*yellow*) transplanted in the SVZa at 6 weeks after transplantation. *A*, Vimentin (*VIM*) staining delineated the RMS. BrdU-positive cells (*arrows*) were observed along the RMS but were not VIM positive. *B*, Many host cells present within the RMS were positively stained with an antibody to Hu, a neuronal phenotypic marker, and some transplanted cells were also Hu positive (*arrowhead*, enlarged in the *inset*). *C*, Many Hu-positive transplanted cells (*arrowheads*) were located within the granule cell layer of the olfactory bulb. The *inset* shows two Hu-positive, BrdU-labeled cells (one strongly and one weakly BrdU labeled). *D*, Approximately half of the transplanted cells (*arrowheads*) were double-labeled with NeuN in both the granule cell layer (*D*) and the periglomerular layer (*E*). *G*, Glomerulus. *Insets* in *D* and *E* show double-labeled cells in higher magnification. *F*, *G*, A small proportion of the BrdU-positive cells found in the periglomerular layer were also TH positive (*arrowheads*). Scale bar (shown in *A*): *A–F*, 100 μm; *G*, 50 μm.

Table 1. The proportion of BrdU-labeled cells, which also express other markers of mature CNS phenotypes, at 6 weeks after transplantation to different regions of the adult rat brain

	SVZa and RMS transplants			
	Graft core	RMS	Granular layer	Periglomerular layer
Hu	0	+	++++	+++
β-tubulin-III	0	+	–	–
NeuN	0	0	+++	++
GAD_{67}	0	0	+++	++
TH	0	0	–	+
DARPP-32	0	0	0	0
GFAP	0	0	0	0
Vimentin	0	0	0	0

	Hippocampal transplants				
	Graft core	Subgranular layer	Granule cell layer	Hilus	CA3
Hu	++	+++++	++++	+	0
β-tubulin-III	++	+++++	++++	–	0
NeuN	+	+++++	++++	0	0
GAD_{67}	–	0	0	–	–
TH	0	0	0	0	0
DARPP-32	0	0	0	0	0
Calbindin	+	+++++	++++	0	0
GFAP	++	0	0	++	++

	Striatal transplants		
	Graft core	<0.4 mm from core	0.4–1.5 mm from core
Hu	+++++	++++	0
NeuN	0	0	0
GAD_{67}	+++	++	+
TH	0	0	0
DARPP-32	0	+	0
Calbindin	0	+,	0
GFAP	++	+++	+++

The frequency of double-labeled cells was quite consistent for the different phenotypic markers among the animals used for this analysis: olfactory bulb, $n = 4$; hippocampus, $n = 10$; striatum, $n = 10$. Double-labeling was assessed by confocal microscopy of randomly selected areas in sections stained with a combination of FITC-, Cy2-, and Cy3-labeled antibodies (see Materials and Methods). The data are based on observations from three representative animals in each group. 0, Cells not found; +, 1–10%; ++, 11–40%; +++, 41–60%; ++++, 61–80%; +++++, >80%.

The hippocampus

Transplants of 100,000 cells were placed within the hilar region of the dentate gyrus. At both 2 and 6 weeks after grafting, many of the injected BrdU-positive cells remained as a cluster just below the granule cell layer (Fig. 5*A*). This position of the cell deposit is characteristic for cells that are implanted by passive injection into the dentate gyrus, because of the presence of a cleavage plane underneath the granule cell layer (Wells et al., 1988). A significant proportion of the BrdU-positive cells, however, had migrated within the subgranular layer of the dentate gyrus and into the granule cell layer itself (Fig. 5*D–F*). In addition, some cells were found scattered in the hilus and the molecular layer of the dentate gyrus, as well as in the overlying CA3 region. The extent of cell migration was similar at 2 and 6 weeks. Typically, cells that had migrated longer distances from the transplant core were more weakly labeled with BrdU, suggesting that the migrated cells had undergone further cell divisions.

The BrdU-labeled cells that had integrated into the granular and subgranular layers had the same size and shape as the intrinsic host granule cells, and a large number of them expressed the neuronal markers Hu (Fig. 5*B*), NeuN (Fig. 5*C*), and β-tubulin-III (Fig. 5*D*) at both time points. The calbindin marker that is characteristic for the intrinsic granule cells was clearly present in many of the transplanted cells at 6 weeks but not at 2 weeks after transplantation. Occasional BrdU/Hu double-labeled cells, but no BrdU/NeuN or BrdU/calbindin double-labeled cells, were found outside these layers. A large proportion of the transplanted cells within the granule cell layer were calbindin positive (Fig. 5*E*). No BrdU/GAD_{67} double-labeled cells were observed in these transplants (Fig. 5*F*). Similarly, no cells that coexpressed BrdU and DARPP-32 were observed within any region of the hippocampus. BrdU-labeled cells expressing the glial marker GFAP were found in areas outside the dentate gyrus, both in the CA3 area and in areas close to ventricle as well as within or close to the graft core (see Fig. 9*C*). The extent of neuronal and glial differentiation of the transplanted cells within each region of the hippocampus is given in Table 1.

Staining with the hTau antibody revealed scattered axonal and cellular profiles, both within the graft core and in individual cells that had migrated away from the initial transplant site within the

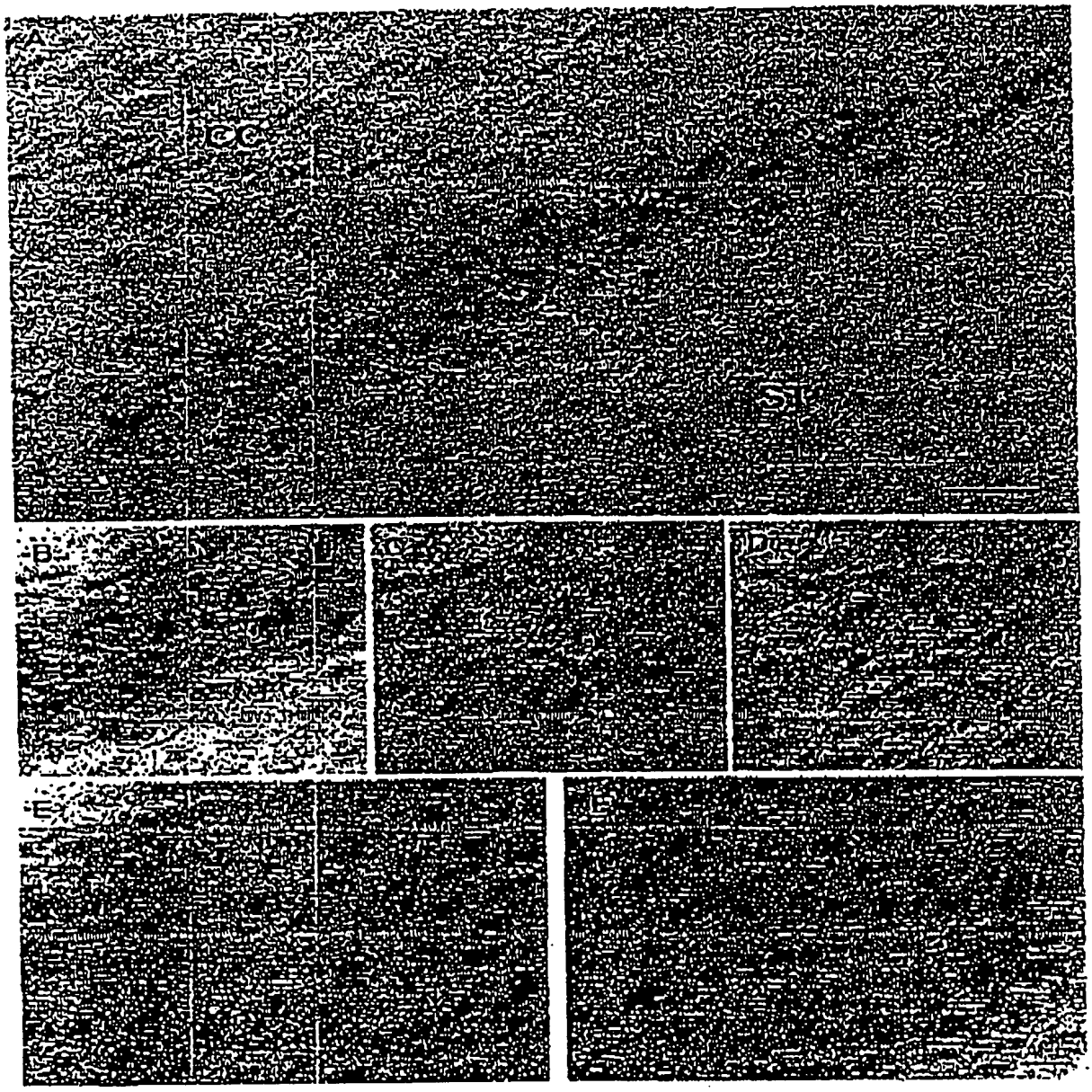

Figure 3. Grafts to the SVZa stained with a human-specific antibody to tau, at 6 weeks after transplantation. *A*, Sagittal section showing neuronal cell bodies and axons located within the SVZa, between the striatum (*ST*) and the overlying corpus callosum (*CC*). *B–D*, Higher magnification of individual neuronal profiles at the periphery of the transplant (*B*) and migrating in the RMS (*C*, *D*). *E*, *F*, Individual cells located deep within the olfactory bulb, showing morphological features of mature neurons. Scale bars: *A*, 100 μm; (shown in *F*) *B–F*, 10 μm.

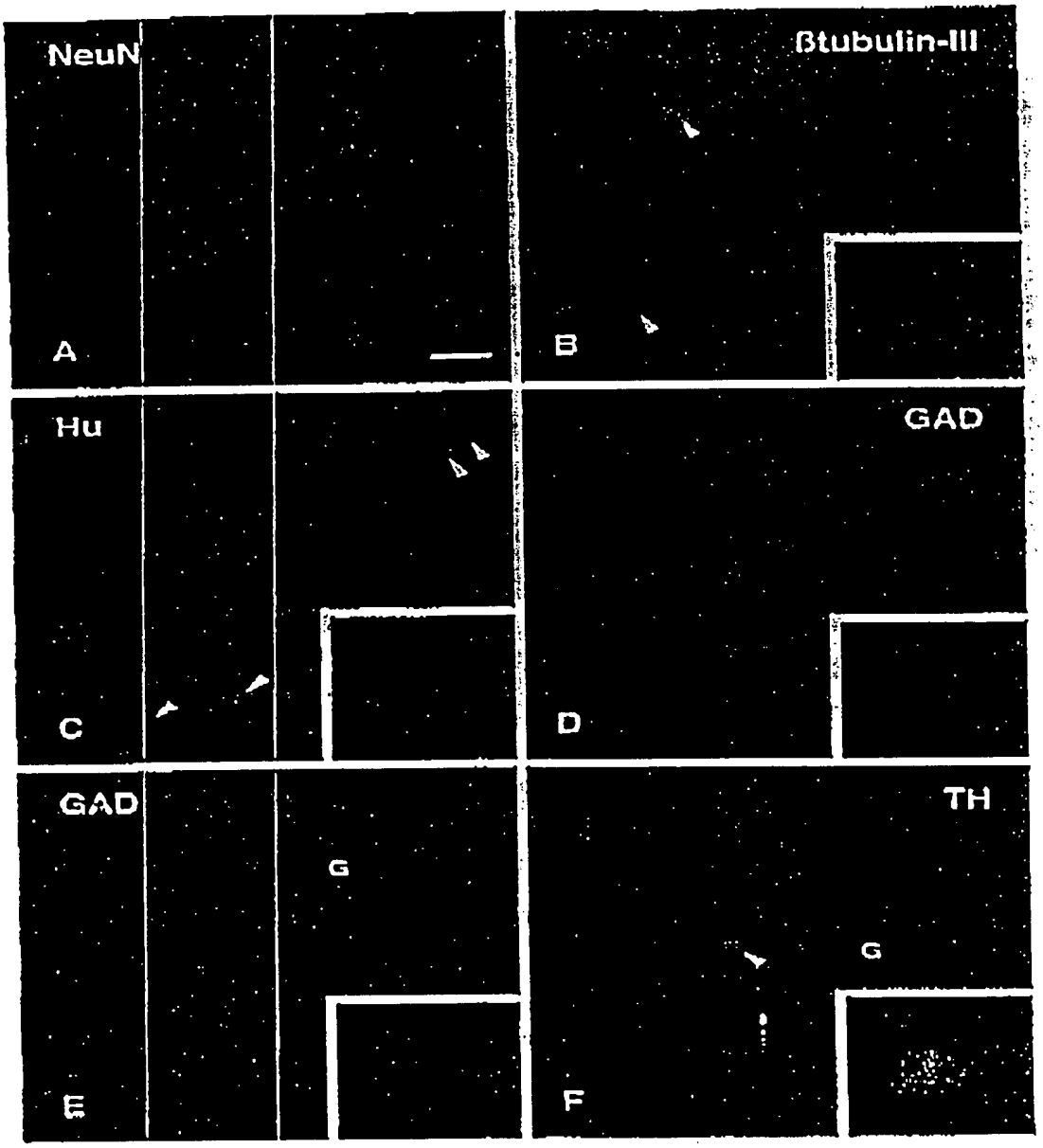

Figure 4. Confocal images of BrdU-labeled (*green*) and double-labeled cells (*yellow*) transplanted in the RMS. *A*, A typical graft at 2 weeks after transplantation, situated in the RMS with only moderate migration of grafted cells in either rostral and caudal direction from the graft core. *B*, At 6 weeks after transplantation cells were seen migrating along the RMS. A small proportion of the transplanted cells were labeled with the early neuronal marker β-tubulin III (*arrowheads*). This marker was also present in many of the host cells within this pathway. *C*, Transplanted cells were found scattered throughout the olfactory bulb. Many of them were Hu positive (*arrowheads*), indicating their differentiation to a neuronal phenotype. *D*, Within the granule cell layer many of the BrdU-labeled cells were GAD_{67} positive. Within the periglomerular layer, BrdU-positive cells stained positively for either GAD_{65} (*E*) or TH (*F*). *Insets* show individual double-labeled cells in higher magnification. *Arrow* in *F* marks a TH-negative transplanted cell. Scale bar (shown in *A*): *A*, 150 μm; *B*, *C*, 50 μm; *D–F*, 25 μm.

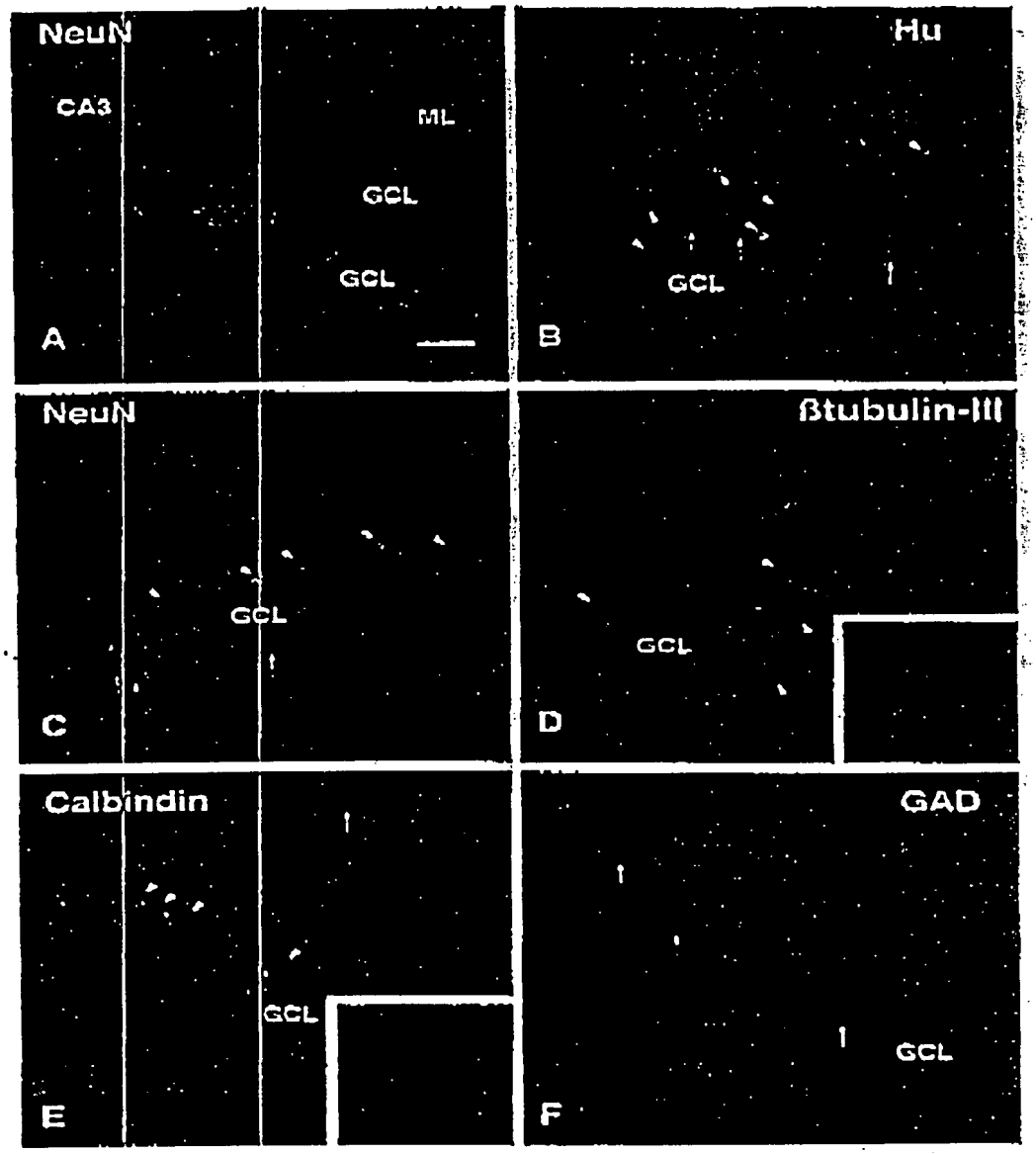

Figure 3. Confocal images of BrdU-labeled (*green*) and double-labeled cells (*yellow*) transplanted to the hippocampus. *A*, The core of transplanted cells was located within either the dorsal or ventral blades of the granule cell layer (*GCL*) in the dentate gyrus. *B*, *C*, By 2 weeks after transplantation, Hu- and NeuN-positive BrdU-labeled cells were observed at some distance from the graft core, mainly in the subgranular layer and also within the granule cell layer (*arrowheads*). *D*, BrdU-labeled transplanted cells positive for the neuronal marker β-tubulin-III (*arrowheads*) were found both within the graft core and in cells that had migrated along the subgranular layer. *E*, At 6 weeks (but not at 2 weeks) after transplantation, calbindin-positive cells were observed in the granule cell layer (*arrowheads*). *F*, No GAD_{67}-positive interneurons were observed. *Insets* show an individual β-tubulin-III/BrdU-labeled cell in the cluster of grafted cells in the subgranular layer in *D*, and two calbindin/BrdU-labeled cells within the deep part of the granule cell layer. *Arrows* indicate single-labeled BrdU-positive cells. Scale bar (shown in *A*): *A*, 150 μm; *B*, *C*, 75 μm; *D–F*, 50 μm. *CA3*, CA3 region of hippocampus; *ML*, molecular layer; *GCL*, granule cell layer.

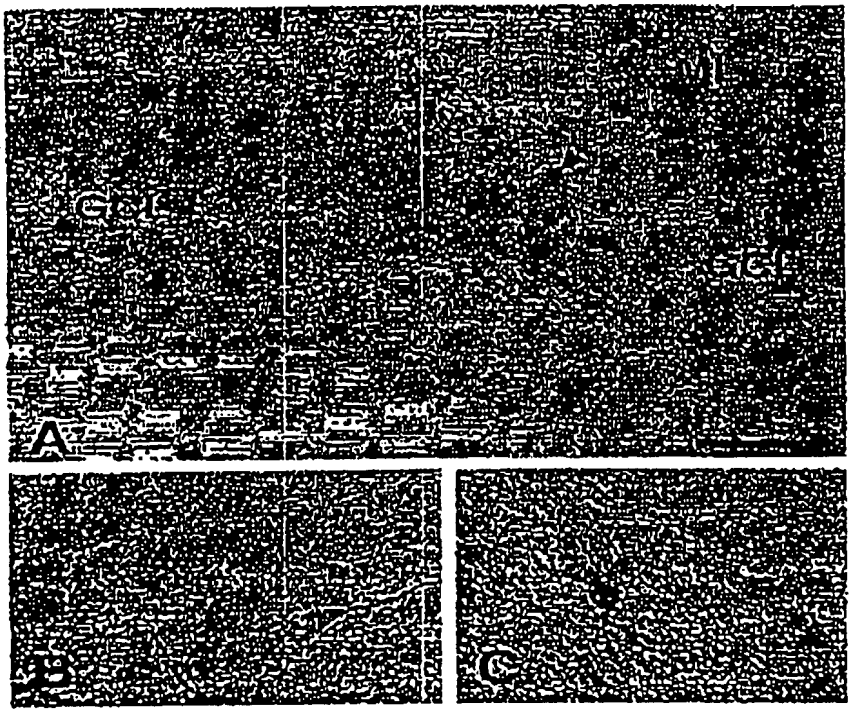

Figure 6. Hippocampal transplants stained with the hTau antibody. *A*, A transplant in the dentate gyrus at 6 weeks after transplantation. *B*, A tau-positive cell with a typically immature neuronal profile with one primary process. *C*, A more differentiated tau-positive neuron with more complex processes, situated within the subgranular layer (*arrowhead* in *A*). Scale bar (shown in *A*): *A*, 150 μm; *B*, *C*, 30 μm. *ML*, Molecular layer; *GCL*, granule cell layer; *H*, hilus.

granular and subgranular cell layers (Fig. 6*A*). At 2 weeks the cells appeared fairly immature, with a few short Tau-positive processes. At 6 weeks, cells with morphological features of neurons with processes were observed (Fig. 6*B*,*C*). Tau-positive cells were also seen in the hilus and molecular layer and along the needle tract.

The striatum

The transplants were placed centrally within the head of the caudate-putamen. At both 2 and 6 weeks after grafting, the grafted cells were found as a BrdU-labeled cell cluster at the site of implantation. Many of the BrdU-labeled cells, however, were observed to have migrated into the surrounding host striatum, without any preferential direction, to a distance of ~1–1.5 mm from the graft core (Fig. 7*A*). The size of individual BrdU nuclei varied considerably, both within the graft core and in cells that were located in the adjacent host striatum (<0.4 mm from the graft core). All of the cells that had migrated over longer distances were of small size and more faintly labeled, suggesting a dilution of the BrdU label caused by cell division. In sagittal sections the BrdU-positive cells could be seen to be aligned with the gray matter, interspersed with the fibers of the internal capsule.

Double-staining revealed that the majority of the BrdU-positive cells in the graft core and in the adjacent host striatum were double-labeled for the early neuronal marker Hu (Fig. 7*B*) but negative for NeuN (Fig. 7*C*). Many BrdU/Hu double-labeled cells occurred also at the graft-host border and within the adjacent host striatum, up to a distance of ~0.3–0.4 mm from the graft core. Although the majority of the Hu-positive cells within the graft core were small in size and round or oval in shape, similar to the Hu-positive cells within the SVZ of the host brain, a substantial proportion of the BrdU/Hu-positive cells at the graft-host border and in the host striatum were larger in size (10–15 μm), i.e., in the range of the Hu-positive neurons within the host striatum. None of the cells expressed NeuN, which is also the case, however, for most of the host striatal neurons. All BrdU-labeled cells located farther away from the graft core were Hu negative. These cells were all of small size and often found in satellite positions, closely apposed to host striatal neurons (Fig. 7*C*, *arrows*) or close to blood vessels. The location and staining properties of these small-sized cells suggest that they had differentiated, at least in part, into glia. Colocalization of BrdU and the astrocyte marker GFAP was unequivocally demonstrated at the graft-host border, i.e., within the area of GFAP-positive reactive astrocytes surrounding the graft core (see Fig. 9*D*, *inset*).

The neuronal phenotype of the transplanted cells was further investigated using antibodies against the GABA-synthesizing enzyme GAD_{67}, which is present in the vast majority (>90%) of the neurons within the striatum; DARPP-32, which is a marker for the medium-sized spiny striatal projection neurons; and calbindin, which is normally present in the medium spiny projection neurons in the matrix component of the striatum (for review, see Gerfen, 1992). BrdU/GAD_{67} double-labeled cells were observed both in the transplant core and within the host striatum at the

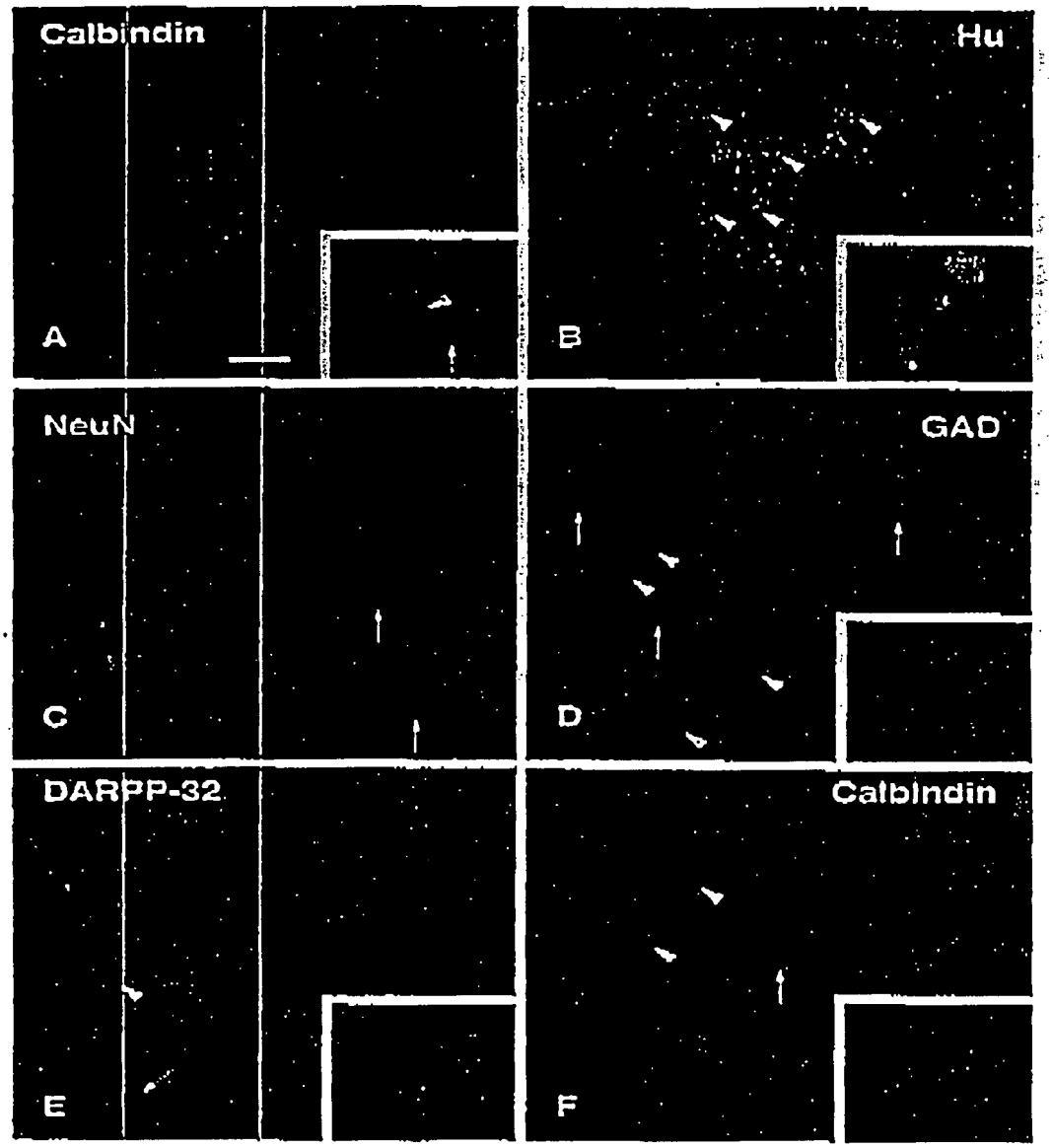

Figure 7. Confocal images of BrdU-labeled (*green*) and double-labeled cells (*yellow*) transplanted in the striatum. *A*, Coronal section through the graft core at 6 weeks after transplantation, showing a dense cluster of cells at the injection site and migration of BrdU-labeled cells away from the graft core, in both gray and white matter. *Inset* shows region in *box* at higher magnification, also illustrated in *F*. *B*, Many of the transplanted cells were positively stained with Hu (*red*), even within the graft core (*arrowheads*, enlarged in the *inset*). *C*, No BrdU/NeuN double-labeled cells were found in the graft core or among those cells that had migrated into the host striatum. *Arrows* indicate transplanted cells that were found in close association with NeuN-positive host neurons (*red*). *D*, A number of transplanted cells were positive for the enzyme GAD_{67} in the periphery of the graft core (*arrowheads*). One of the double-labeled cells is shown at higher magnification in the *inset*. *E*, BrdU/DARPP-32 double-labeled cells were occasionally observed (*arrowhead* and *inset* at higher magnification). These were generally faintly labeled and found only in the immediate vicinity of the transplant core. *F*, Similarly, BrdU/Calbindin double-labeled cells were found both in the periphery of the graft and in adjacent regions of the host striatum. Scale bar (shown in *A*): *A*, 300 μm; *B*, 150 μm; *D–F*, 25 μm.

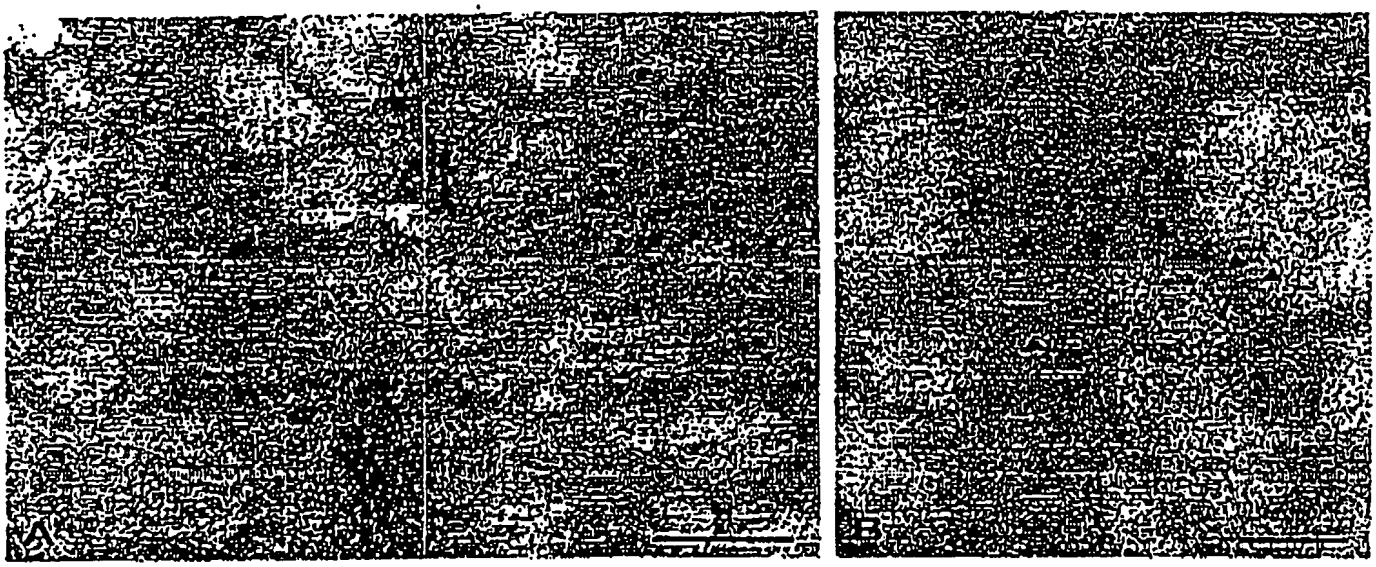

Figure 8. Striatal transplants stained with the hTau antibody. Six weeks after transplantation, coronal sections revealed tau-positive neuronal profiles densely packed within the graft core (*A*). Individual cells with neuronal profiles were observed also in the host striatum adjacent to the graft (*arrowheads* in *A* and *B*). Axonal processes were seen to extend caudally within the white matter bundles of the internal capsule (*arrow* in *A* and *B*). Scale bars: *A*, 500 μm; *B*, 100 μm.

periphery of the transplants (Fig. 7*D*). In addition, some BrdU-labeled cells expressed calbindin (Fig. 7*A,F*, *inset*) and occasionally also DARPP-32. These cells were located at the periphery of the transplants and in the adjacent host striatum up to a distance of ~0.3–0.4 mm from the graft-host border and were similar in size and shape to those present within the host striatum. The BrdU/DARPP-32 double-labeled cells were only weakly DARPP-32 positive but were comparable in size to the host DARPP-32-positive neurons (Fig. 7*E*). None of the transplanted cells expressed TH, either within the graft core or within the host striatum. Table 1 outlines the extent of expression of neuronal and glial markers at different distances from the graft core.

Staining with the hTau antibody revealed a graft core of clustered tau-positive cells and fibers (Fig. 8*A*). In sagittal sections, loose bundles of tau-positive fibers were seen to leave the graft core in both the rostral and caudal direction, along the white matter bundles of the internal capsule. In cross section, these fibers were found primarily within the white matter bundles (Fig. 8*B*, *arrows*). Individual cells were also observed at some distance from the graft core (Fig. 8*A,B*, *arrowheads*). In these cases, the cell bodies were often located within the gray matter, with their processes projecting into the white matter tracts. At 6 weeks, tau-positive axons could be traced caudally from the graft core within the internal capsule bundles for a distance of ~1–2 mm; some of these fibers were seen to enter the globus pallidus, and in some cases scattered tau-positive fibers could be traced as far as the entopeduncular nucleus.

DISCUSSION

These present results show that the long-term propagated human neurosphere cultures contained progenitors that can respond *in vivo* to cues present in both neurogenic and non-neurogenic regions of the adult rat brain. The expression of phenotypic markers provided evidence for site-specific neural differentiation within each of the three grafted regions. In the olfactory bulb the cells that integrated into the granular and periglomerular layers expressed NeuN, TH, and GAD_{67}, similar to the dopaminergic and GABAergic cells normally present in these regions. In the dentate gyrus some of the cells assumed a position, morphology, and phenotype similar to the NeuN/calbindin-positive granule cells within the granule cell layer. And in the striatum, cells located in the periphery of the transplants expressed GAD_{67} and calbindin as well as low levels of the striatum-specific marker DARPP-32.

A combination of EGF, bFGF, and LIF was used to expand the human progenitors. It has been shown previously that EGF and bFGF act cooperatively in promoting the proliferation of rat and human neural progenitors (Vescovi et al., 1993; Weiss et al., 1996a; Svendsen et al., 1997). bFGF appears to be a mitogen for both unipotent and multipotent neuronal and glial progenitors (Murphy et al., 1990; Vescovi et al., 1993; Ray and Gage, 1994; Kilpatrick and Bartlett, 1995; Palmer et al., 1995) and may act broadly to maintain neural progenitor cells as a constitutively proliferating population *in vitro* (Palmer et al., 1995). It seems likely, therefore, that the combination of growth factors used here served to maintain both multipotent and lineage-restricted progenitors in continuous cell cycle and that the ability to migrate and integrate into the adult host brain was expressed by specific subsets of cells. Previous studies suggest that the *in vivo* properties of *in vitro* expanded neural progenitors may differ depending on the growth factors used. Rat or mouse neurosphere cells ex-

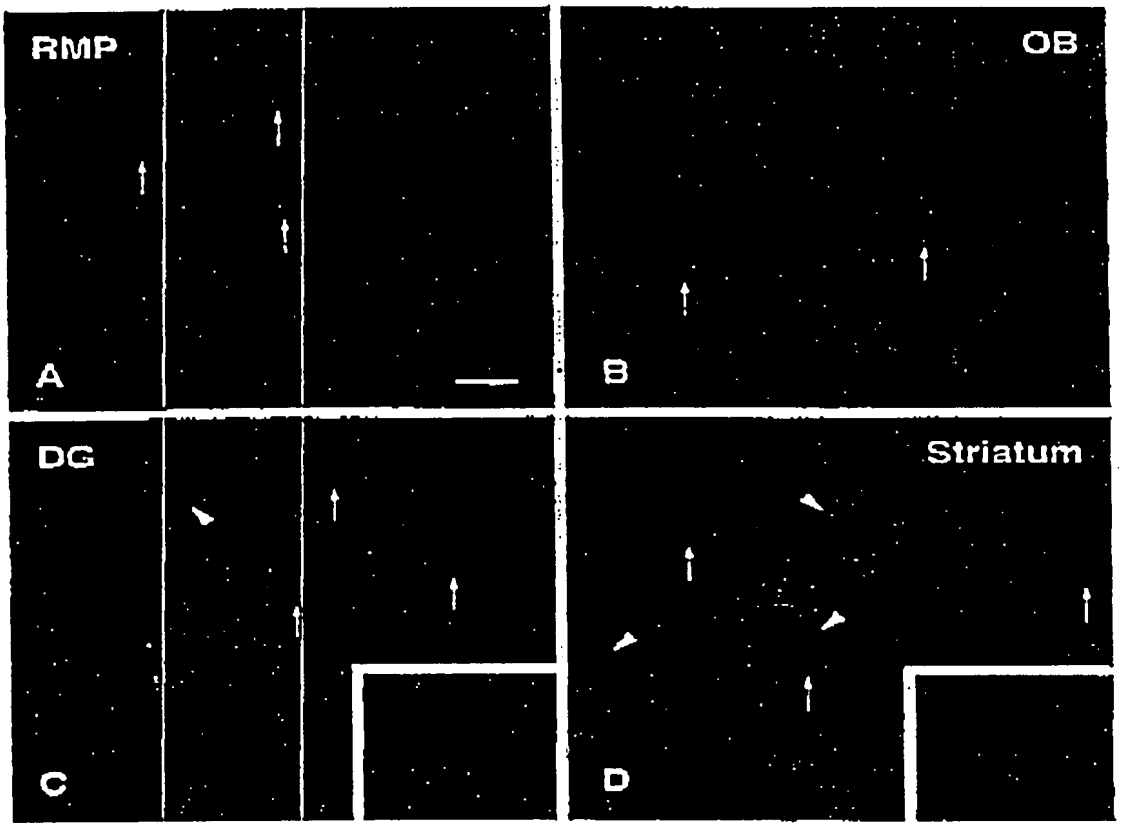

Figure 9. GFAP was used to label astrocytes within the graft areas (*red*) to assess the extent of colocalization with BrdU-labeled transplanted cells (*green*). *A*, Cells within the RMS at 6 weeks after transplantation were often closely associated with GFAP profiles, although no double-labeled cells were observed (*arrows*). *B*, Within the granule cell layer of the olfactory bulb, BrdU-positive cells were interspersed with, but not colocalized with, GFAP (*arrows*). *C*, In the dentate gyrus, cells within the transplant core were occasionally closely associated with GFAP-positive cytoplasmic staining, possibly indicating a double-labeled cell. *D*, Staining within the striatum revealed a dense network of GFAP-positive processes (*red*) intermingled with the BrdU-positive cells (*green*). Many clear examples of double-labeled cells were observed (*arrowheads*), although examples of BrdU single-labeled cells were also frequently observed (*arrows*). *Insets* show BrdU/GFAP double-labeled cells at higher magnification. Scale bar (shown in *A*): *A*, *C*, 50 μm; *B*, 25 μm; *D*, 150 μm.

pended in the presence of EGF alone have generated only glial cells and no neurons after transplantation to the developing rat forebrain (Winkler et al., 1998) or adult rat spinal cord (Hammang et al., 1997), and they exhibit poor survival and integration after transplantation to the striatum (Svendsen et al., 1996; C. Winkler, R. A. Fricker, A. Björklund, unpublished observations). By contrast, adult rat hippocampal progenitors cultured in the presence of bFGF exhibit both migration and neurogenesis after transplantation in the adult rat brain (Gage et al., 1995; Suhonen et al., 1996).

Site-specific differentiation of the grafted cells

In the SVZa, which is one of the two sites where neurogenesis continues into adulthood in the mammalian CNS, the endogenous neuronal progenitors have been shown to migrate along the RMS and reach the bulb within 2–15 d after their generation in the SVZa (Lois and Alvarez-Buylla, 1994). The cells are already committed to a neuronal phenotype while in the migratory path, although they continue to divide during migration (Menezes et al., 1995). The cells generated by SVZa postnatally are interneurons, above all GABAergic and dopaminergic interneurons in the granular and periglomerular layers of the olfactory bulb (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Betarbet et al., 1996).

The transplanted human neural progenitor cells expressed the early neuronal markers Hu and β-tubulin-III during migration to the olfactory bulb, indicating that some of the transplanted progenitors were committed to a neuronal fate already in the SVZ, similar to the endogenous neuronal progenitors generated in the SVZa (Lois and Alvarez-Buylla, 1994; Menezes et al., 1995). On

reaching the bulb, BrdU-positive cells distributed in the granular and periglomerular layers and coexpressed neuronal markers such as Hu and NeuN, as well as hTau. This is in agreement with previous results obtained with rat or mouse SVZa progenitors (Luskin, 1993; Lois and Alvarez-Buylla, 1994) and a recent study using transplantation of human neural stem cells (Flax et al., 1998). One interesting difference between the transplanted human cells in the current study and endogenous SVZa progenitors is the time course of migration: few of the transplanted human progenitors had entered the RMS at 2 weeks, and many still remained dispersed along the RMS by 6 weeks. One reason for this may be a species difference. Transplants of human primary cells show a more protracted development than rat-to-rat grafts, which suggests that the human cells retain some type of internal developmental clock for their differentiation and maturation (Grasbon-Frodl et al., 1996, 1997). Indeed, Suhonen et al. (1996) reported that adult rat neural progenitors transplanted to the SVZa in adult rats are distributed along the entire length of the RMS by 1 week, and by 8 weeks ~90% of the cells had reached the bulb. Similarly, Lois and Alvarez-Buylla (1994) observed that SVZa progenitors, implanted into the adult SVZa, reach the bulb within 30 d after transplantation. These observations indicate that the slow onset and protracted time course of migration of the human cells reflect intrinsic developmental constraints.

In hippocampus the transplanted cells distributed along the subgranular and granular layers of the dentate gyrus. Although cells were observed also in other layers of the dentate and the CA3 region, cells expressing neuronal markers occurred only within the subgranular or granular layers, suggesting that the human progenitors, similar to rat hippocampal and cerebellar progenitors (Gage et al., 1995; Vicario-Abejon et al., 1995), are able to respond to local cues specifically localized in these layers. The transition zone between the hilus and the granule cell layer is the site where endogenous neuronal progenitors are normally generated (Altman and Das, 1966; Altman and Bayer, 1990), providing a source of new granule cells throughout life (Kaplan and Hinds, 1977; Cameron et al., 1993; Kuhn et al., 1996). As judged by morphological criteria, i.e., size, shape, and distribution of the cells, and expression of characteristic neuronal markers, the grafted progenitors are induced by local signals to express neuronal features similar to the resident granule cells. It remains to be demonstrated, however, to what extent these newly formed neurons can undergo complete maturation and establish appropriate axonal and dendritic connectivity.

Cells grafted to the striatum generate both neurons and glia

Expression of neuronal markers in the striatal transplants indicate that a substantial fraction of the grafted human progenitors had developed toward a neuronal phenotype. Many of the Hu-positive cells within the transplant core were small and round or oval in shape, similar to the neuronal precursors normally present in the proliferative subependyma in the adult brain. These cells did not express any of the more mature neuronal markers and therefore may be classified as poorly differentiated neuronal precursors. The GAD_{67}-, calbindin-, and DARPP-32-positive cells were exclusively located at the graft–host border and within the adjacent host striatum, up to a distance of ~0.3–0.4 mm. The size and shape of these cells were similar to the medium-sized neurons of the host striatum. Many of these are GABAergic and stain positively for GAD_{67}; one subclass, the striatal projection neurons, is further characterized by the expression of calbindin and/or DARPP-32.

These data indicate that the human neural progenitors can undergo neurogenesis also in the the normally non-neurogenic environment of the adult striatum and assume neuronal phenotype(s) similar to those normally present here but that in the absence of suitable substrates for migration they remain close to the implantation site. Interestingly, in sections stained with the hTau antibody some of these newly formed neurons were seen to extend long axon-like processes that could be traced along the fascicles of the internal capsule to the globus pallidus and in some cases also the entopeduncular nucleus, a distance of ~2 mm.

The cells that migrated over longer distances within the adult striatum were all Hu negative and of small size. Many of them were found in satellite position to the medium-sized host striatal neurons or close to blood vessels, suggesting that they had assumed a glial-like phenotype (Fig. 9). A migratory capacity of immature glia (or glial precursors) within the adult CNS has been reported for both astrocytes and oligodendrocytes by several investigators (Blakemore and Franklin, 1991). Extensive astrocyte migration within the adult striatum, similar in extent to the one observed here, has previously been described in transplants of human neuronal progenitors (Svendsen et al., 1997) and freshly dissociated human embryonic striatal and diencephalic tissue (Pundt et al., 1995). In these cases the migratory cells appear to be glial precursors in a proliferative, migratory stage of their development. Consistent with this, we observed that cells located at progressively greater distances from the transplant core had lower levels of BrdU labeling than the cells that remained at the implantation site, suggesting that the migrating cells continued to divide as they dispersed within the host striatal parenchyma.

Implications for brain repair

The human neurosphere cultures are particularly suitable for transplantation in that they can be harvested and implanted without dissociation and detachment from a culture substrate. The cultures used here had been expanded up to 10 million-fold, which means that each transplant of 100,000–200,000 cells in theory could be derived from a single cell in the original cell preparation. Because the *in vivo* properties of the cells were indistinguishable over a wide range of passages (from 9 to 21), the present culture system could provide an almost unlimited source of human neural progenitor cells for transplantation.

The present results show that subpopulations of cells contained within the human neurosphere cultures can respond appropriately to specific extracellular cues present in each of the four target regions in the adult rat brain. Because the human neurosphere cultures are likely to contain a mixture of multipotent and lineage-restricted progenitors, the specific migratory patterns seen in the different locations may be explained either by the ability of an undifferentiated stem cell-like cell to differentiate along alternative neuronal or glial pathways in response to diverse local cues, or alternatively, by the presence of different subpopulations of lineage-restricted neuronal or glial precursors that were already committed to specific neuronal or glial fates. The present data seem compatible with both alternatives.

In conclusion, the long-term propagated human neural progenitors described here demonstrate a remarkable capacity for migration, integration, and site-specific differentiation in the adult brain. The growth factor combination used here acted to maintain the progenitors as a constitutively proliferating cell popula-

tion without losing their capacity to respond to those extracellular cues normally present in the adult CNS. With further refinement of the procedure, e.g., by application of cell enrichment and cell sorting techniques, this culture system may provide an almost unlimited source of human neural progenitors at different stages of differentiation and lineage restriction. Such cells will be of great interest both as an experimental tool and as an alternative to primary embryonic brain tissue for intracerebral transplantation.

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File A-61105-8 Atty DJB
Due Date 96.07.19
Type 30 DAY RESP Refs

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

APPLICATION NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
08/479,796	06/07/95	WEISS	S A-61105-8/DJ

EXAMINER: ZISKA, S

18N2/0619
FLEHR HOHBACH TEST ALBRITTON & HERBERT
FOUR EMBARCADERO CENTER SUITE 3400
SAN FRANCISCO CA 94111-4187

ART UNIT	PAPER NUMBER
1804	6

DATE MAILED: 06/19/96

This is a communication from the examiner in charge of your application.
COMMISSIONER OF PATENTS AND TRADEMARKS

OFFICE ACTION SUMMARY

☐ Responsive to communication(s) filed on ____________

☐ This action is **FINAL.**

☐ Since this application is in condition for allowance except for formal matters, **prosecution as to the merits is closed** in accordance with the practice under *Ex parte Quayle*, 1935 D.C. 11; 453 O.G. 213.

A shortened statutory period for response to this action is set to expire ______ month(s), or thirty days, whichever is longer, from the mailing date of this communication. Failure to respond within the period for response will cause the application to become abandoned. (35 U.S.C. § 133). Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

Disposition of Claims

☑ Claim(s) 1-31 is/are pending in the application.

Of the above, claim(s) ____________ is/are withdrawn from consideration.

☐ Claim(s) ____________ is/are allowed.

☐ Claim(s) ____________ is/are rejected.

☐ Claim(s) ____________ is/are objected to.

☑ Claims 1-31 are subject to restriction or election requirement.

Application Papers

☐ See the attached Notice of Draftsperson's Patent Drawing Review, PTO-948.

☐ The drawing(s) filed on ____________ is/are objected to by the Examiner.

☐ The proposed drawing correction, filed on ____________ is ☐ approved ☐ disapproved.

☐ The specification is objected to by the Examiner.

☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. § 119

☐ Acknowledgement is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).

☐ All ☐ Some* ☐ None of the CERTIFIED copies of the priority documents have been

☐ received.

☐ received in Application No. (Series Code/Serial Number) ____________.

☐ received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

*Certified copies not received: ____________.

☐ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. § 119(e).

Attachment(s)

☐ Notice of Reference Cited, PTO-892

☐ Information Disclosure Statement(s), PTO-1449, Paper No(s). ______

☐ Interview Summary, PTO-413

☐ Notice of Draftsperson's Patent Drawing Review, PTO-948

☐ Notice of Informal Patent Application, PTO-152

Restriction to one of the following inventions is required under 35 U.S.C. § 121:

I. Claims 1-8, drawn to a method for the in vitro proliferation of a multipotent neural stem cell comprising the steps of obtaining the neural tissue, dissociating the neural tissue, culturing the neural tissue and passaging the neural stem cell progeny, and the cell culture obtained by the method, classified in Class 435, subclass 240.2, for example.

II. Claims 9-15, drawn to a method of producing a cell culture comprising non-tumorigenic, genetically modified neural cells and the cell culture produced, classified in Class 435, subclass 172.3, and Class 435, subclass 240.2, for example.

III. Claims 16-18, drawn to a method of remyelinating a neuron comprising dissociating neural tissue, exposing the dissociated cells to growth factors, harvesting neural stem cell progeny and causing the neural stem cell progeny to come into contact with a demyelinated axon to effect remyelination, classified in Class 424, subclass 93.1, for example.

IV. Claim 19, drawn to a method for the in vivo proliferation of a precursor cell located in the CNS comprising administering at least one proliferation inducing growth factor to the CNS tissue, classified in Class 514, subclass 2, for example.

V. Claims 20-21, drawn to a method for the in vivo genetic modification of a CNS precursor cell located in tissue lining a CNS ventricle comprising administering genetic material, classified in Class 514, subclass 44, for example.

VI. Claims 22-25, drawn to a method of treating a neurological disorder comprising administering a composition comprising a proliferation inducing growth factor, classified in Class 514, subclass 2, for example.

VII. Claims 26-27, drawn to a method of transplanting neural stem cell progeny, classified in Class 424, subclass 93.1, for example.

VIII. Claims 28-30, drawn to a method for determining the effect of at least one biological agent on the differentiation of neural cells, classified in Class 435, subclass 7.1, for example.

IX. Claim 31, drawn to a cDNA library, classified in Class 536, subclass 23.1, for example.

The inventions are distinct, each from the other because of the following reasons:

Invention I is independent and distinct from Inventions II-IX since the method of I is drawn to in vitro culture of neural stem cells and does not require the genetic modification of the cells as does Invention II. Invention III is independent from all of the listed inventions since III requires contacting the neural stem cell progeny with a demyelinated axon and therefore the method requires different procedures and different compositions than does either of Inventions I or II. Invention IV is drawn to a method for the in vivo proliferation of a precursor cell and therefore requires different protocols and starting materials than does any of Inventions I-III. Invention V is independent and distinct from Inventions I-IV since V requires the in vivo genetic modification of the cells in situ and therefore requires different starting materials not required by any of Inventions I-IV. Invention VI is independent and distinct from any of Inventions I-V since VI requires the in vivo use of a proliferating inducing factor, usually a protein, and therefore the starting materials and protocols are different than in any of Inventions I-V. Invention VII is independent and distinct from any of Inventions I-VI since VII requires the transplantation of

cells and therefore requires use of different protocols than does any of inventions I-VI. Invention VIII is independent and distinct from any of Inventions I-VIII since VIII is a method drawn to determining the effect of biological agents and therefore use different procedures and starting materials and end points than does any of Inventions I-VII. Invention IX is independent and distinct from any of Inventions I-VIII since IX is a product not used in any of the above methods. Each of Inventions I-IX has separate search requirements not overlapping other Inventions.

Because these inventions are distinct for the reasons given above and have acquired a separate status in the art as evidenced by their different classification, divergent subject matter and separate search requirements, restriction for examination purposes as indicated is proper.

Applicant is advised that the response to this requirement to be complete must include an election of the invention to be examined even though the requirement be traversed.

Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 C.F.R. § 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a diligently-filed petition under 37 C.F.R. § 1.48(b) and by the fee required under 37 C.F.R. § 1.17(h).

Papers related to this application may be submitted to Group 1800 by facsimile transmission. Papers should be faxed to Group 1800 via the PTO FAX center located in Crystal Mall 1. The faxing of such papers must conform with the notice published in the Official Gazette, 1096 OG (30 November 15, 1989). The CM1 Fax Center number is (703) 308-4227.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Examiner Suzanne Ziska, Ph.D., whose telephone number is (703)308-1217. In the event the examiner is not available, the examiner's supervisor, Ms. Jacqueline Stone, may be contacted at phone number (703) 308-3153.

SUZANNE E. ZISKA
PRIMARY EXAMINER
GROUP 1800

File A-61105-1/Atty DSB
Due Date 96-09-13
Type 3b Day Resp Refs

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
08/486,313	06/07/95	WEISS	A-61105-1/D

EXAMINER	
JONES, E	
ART UNIT	PAPER NUMBER
1804	8

DATE MAILED: 08/14/96

18N2/0814

FLEHR HOHBACH TEST ALBRITTON
AND HERBERT
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SUITE 3400
SAN FRANCISCO CA 94111

This is a communication from the examiner in charge of your application.
COMMISSIONER OF PATENTS AND TRADEMARKS

☒ This application has been examined ☐ Responsive to communication filed on _____ ☐ This action is made final.

For Restriction purposes only

A shortened statutory period for response to this action is set to expire ~~_____ month~~(s), 30 days from the date of this letter.
Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

Part I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. ☐ Notice of References Cited by Examiner, PTO-892.
2. ☐ Notice of Draftsman's Patent Drawing Review, PTO-948.
3. ☐ Notice of Art Cited by Applicant, PTO-1449.
4. ☐ Notice of Informal Patent Application, PTO-152.
5. ☐ Information on How to Effect Drawing Changes, PTO-1474.
6. ☐ _____.

Part II SUMMARY OF ACTION

1. ☒ Claims 1 - 31 are pending in the application.

   Of the above, claims _____ are withdrawn from consideration.

2. ☐ Claims _____ have been cancelled.
3. ☐ Claims _____ are allowed.
4. ☐ Claims _____ are rejected.
5. ☐ Claims _____ are objected to.
6. ☒ Claims 1 - 31 are subject to restriction or election requirement.
7. ☐ This application has been filed with informal drawings under 37 C.F.R. 1.85 which are acceptable for examination purposes.
8. ☐ Formal drawings are required in response to this Office action.
9. ☐ The corrected or substitute drawings have been received on _____. Under 37 C.F.R. 1.84 these drawings are ☐ acceptable; ☐ not acceptable (see explanation or Notice of Draftsman's Patent Drawing Review, PTO-948).
10. ☐ The proposed additional or substitute sheet(s) of drawings, filed on _____. has (have) been ☐ approved by the examiner; ☐ disapproved by the examiner (see explanation).
11. ☐ The proposed drawing correction, filed _____, has been ☐ approved; ☐ disapproved (see explanation).
12. ☐ Acknowledgement is made of the claim for priority under 35 U.S.C. 119. The certified copy has ☐ been received ☐ not been received ☐ been filed in parent application, serial no. _____; filed on _____.
13. ☐ Since this application apppears to be in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11; 453 O.G. 213.
14. ☐ Other

EXAMINER'S ACTION

Part III DETAILED ACTION

Election/Restriction

1. Restriction to one of the following inventions is required under 35 U.S.C. 121:

Group I. Claims 1-8, drawn to a method for the in vitro proliferation of a multipotent neural stem cell comprising the steps of obtaining the neural tissue, dissociating the neural tissue, culturing the neural tissue, and passaging the neural stem cell progeny, and the cell culture obtained by the method , classified in Class 435, subclass 240.2, for example.

Group II. Claims 9-15, drawn to a method of producing a cell culture comprising non-tumorgenic, genetically modified neural stem cells and the cell culture produced, classified in Class 435, subclass 172.3, and class 435, subclass 240.2, for example.

Group III. Claims 16-18, drawn to a method of remyelinating a neuron comprising dissociating neural tissue, exposing dissociated neural tissue to growth factors, harvesting the neural stem cell progeny and causing the neural stem cell progeny to come into contact with a demyelinated axon to effect remyelination as classified in Class 424, subclass 93.1, for example.

Group IV. Claim 19, drawn to a method for the in vivo proliferation of a precursor cell located in the CNS comprising administering at least one proliferation inducing growth factor two the CNS tissue, classified in Class 514, subclass 2 for example.

Group V. Claims 20-21, drawn to the method for the in vivo genetic modification of a CNS precursor cell located on tissue lining a CNS ventricle comprising administering genetic material and at least one proliferation inducing growth factor to said ventricle, classified in Class 514, subclass 44, for example.

Group VI. Claims 22-25, drawn to a method of treating a neurological disorder comprising administering a composition comprising a proliferation inducing growth factor and genetic material, classified in Class 514, subclass 2 and Class 514, subclass 44, for example.

Group VII. Claims 26-27, drawn to method of transplanting neural stem cell progeny, classified in Class 424, subclass 93.1, for example.

Group VIII. Claims 28-30, drawn to a method for determining the effect of at least one biological agent on the differentiation of neural cells, classified in Class 435, subclass 7.1, for example.

Group IX. Claim 31, drawn to a cDNA library, classified in Class 536, subclass 23.1, for example.

The inventions are distinct, each from the other because of the following reasons:

Invention I is independent and distinct from Inventions II-IX since the method of I is drawn to in vitro culture of neural stem cells and does not require the genetic modification of the cells as does invention II. Invention III is independent from all of the listed inventions since III requires contacting the neural stem cell progeny with a demyelinated axon and therefore the method requires different procedures and different compositions than does either of Inventions I or II. Invention IV is drawn to a method for the in vivo proliferation of a precursor cell and therefore requires different protocols and starting materials than does any of Inventions I-III. Invention V is independent and distinct from Inventions I-IV since V requires the in vivo genetic modification of the cells in situ and therefore requires different starting materials not required by any Inventions I-IV. Invention VI is independent and distinct from Inventions I-V since VI requires the in vivo use of a proliferating inducing factor, usually a protein, and or use of genetic material, to treat a neurological disorder and therefore the starting materials and protocols are different than in any of Inventions I-III; and the combination therapy involves materially different

considerations of administration, versus Inventions IV and V. Invention VII is independent and distinct from any of Inventions I-VI since VII requires the transplantation of cells and therefore requires use of different protocols than does any of Inventions I-VI. Invention VIII is independent and distinct from any of Inventions I-VII since VIII is a method drawn to determining the effect of biological agents and therefore use different procedures and starting materials and has different end points than does any of Inventions I-VII. Invention IX is independent and distinct from any inventions I-VIII since IX is a product not used in any of the above methods. Each of Inventions I-IX has separate search requirements which are not coextensive to each of the other Inventions.

Because these inventions are distinct for the reasons given above and have acquired a separate status in the art as evidence by their different classification, divergent subject matter and separate search requirements, restriction for examination purposes as indicated is proper.

Applicant is advised that the response to this requirement to be complete must include an election of the invention to be examined even though the requirement be traversed.

Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 C.F.R. § 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a diligently-filed petition under 37 C.F.R. § 1.48(b) and by the fee required under 37 C.F.R. § 1.17(h).

Papers related to this application may be submitted to Group 1800 by facsimile transmission. Papers should be faxed to Group 1800 via the PTO FAX center located in Crystal Mall 1. The faxing of such papers must conform with the notice published on the Official Gazette, 1096 OG (30 November 15, 1989). The CM1 Fax Center Number is (703) 308-0294.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Ernest Jones whose telephone number is (703) 305-7018. -In the event that the examiner is not available, the examiner's supervisor, Ms. Jacqueline Stone, may be contacted at (703) 308-3153.

Any inquiry of a general nature or relating to the status of this application should be directed to the Group receptionist whose telephone number is (703) 305-0196.

JACQUELINE M. STONE
SUPERVISORY PATENT EXAMINER
GROUP 1800